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CONTENTS

The Longitudinal Vertebral Venous Sinuses of the Dog. I. Anatomy 341
R. P. Worthman

The Longitudinal Vertebral Venous Sinuses of the Dog. II. Functional Aspects 349
R. P. Worthman

Macroscopic Anatomy of the Ear of the Dog 364
R. Getty, H. L. Foust, E. T. Presley, and M. E. Miller

Some Observations on Transplantation of the Canine Kidney 376
J. Archibald and A. J. Cawley

Some Aspects of Infectious Canine Hepatitis Virus in Tissue Culture 380
A. H. Fieldsteel

Studies on Experimental Canine Interstitial Nephritis. I. Evaluation of Certain Nephrotoxic Agents 389
C. E. Stevens, J. J. Clark, and A. F. Sellers

Serological Studies of Canine Distemper—Complement Fixation with Spleen Antigens 398
L. Kilham

Studies of the Bovine Adrenal Gland. I. The Production of Lipid Accumulation and Other Histological and Cytological Changes in the Zonae Glomerulosae of Calves .. 402
A. F. Weber, W. R. Pritchard, and A. F. Sellers

Observations on Collagen, Vitamin A, and Ascorbic Acid in Bovine Hyperkeratosis 410
C. L. Marsh, C. Olson, Jr., and I. C. Blore

Vitamin A Deficiency and Urolithiasis in Range Cattle 415
K. F. Swingle and H. Marsh

Studies on Trichloroethylene-Extracted Feeds. I. Experimental Production of Acute Aplastic Anemia in Young Heifers 425
W. R. Pritchard, C. E. Rehfeld, N. S. Mizuno, J. H. Sautter, and M. O. Schultze

II. The Effect of Feeding Different Levels of Trichloroethylene-Extracted Soybean Oil Meal to Young Heifers—Experimental Production of Chronic Aplastic Anemia 430
W. R. Pritchard, C. E. Rehfeld, W. E. Mattson, J. H. Sautter, and M. O. Schultze

(Continued on page 337)

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(Contents continued from cover)

III. The Use of Young Calves for Study of Various Aspects of Toxicity of Trichloroethylene-Extracted Soybean Oil Meal..... 437
W. R. Pritchard, W. E. Mattson, J. H. Sautter, and M. O. Schultze

IV. Susceptibility of the Horse to the Toxic Factor in Trichloroethylene-Extracted Soybean Oil Meal..... 441
W. R. Pritchard, R. Hammer, J. H. Sautter, and M. O. Schultze

V. Failure to Demonstrate the Presence of a Toxic Factor in the Milk of Cows Fed Toxic Specimens of Trichloroethylene-Extracted Soybean Oil Meal..... 444
W. R. Pritchard, W. E. Mattson, J. H. Sautter, and M. O. Schultze

VI. The Effects of Feeding Trichloroethylene-Extracted Soybean Oil Meal to Sheep 446
W. R. Pritchard, V. Perman, W. E. Mattson, J. H. Sautter, and M. O. Schultze

VII. Observations with Laboratory Animals Fed Trichloroethylene-Extracted Soybean Oil Meal..... 448
W. R. Pritchard, F. Sauer, C. E. Rehfeld, V. Perman, J. H. Sautter, S. Wada, and M. O. Schultze

Research on Foot-and-Mouth Disease. X. The Titration of Cultivated Foot-and-Mouth Disease Virus (Frenkel) in Cattle Compared with the Titer Obtained in the Quantitative Complement-Fixation Test..... 455
H. H. J. Frederiks

Sulfaquinoxaline and Sulfamerazine in the Treatment of Experimental Infections with *Eimeria Bovis* in Calves..... 463
D. M. Hammond, J. L. Shupe, A. E. Johnson, P. R. Fitzgerald, and J. L. Thorne

Experimental Leptospirosis in Pregnant Swine..... 471
L. C. Ferguson and T. E. Powers

Studies on Infectious Atrophic Rhinitis. V. Concept that Several Agents May Cause Turbinate Atrophy..... 478
W. P. Switzer

Effect of Repeated Freezing and Thawing on the Electrophoretic Pattern of Swine Serum 485
J. Mathews and D. A. Buthala

Anaplasma Ovis in the United States. Experimental Studies with Sheep and Goats.... 487
E. J. Splitter, H. D. Anthony, and M. J. Twiehaus

Clostridium Perfringens Type B from Enterotoxemia in Young Ruminants..... 492
F. W. Frank

Studies on the Etiological Agent of Epididymitis in Rams..... 495
M. E. Meyer and H. S. Cameron

Effects of Dried Preserved Rumen Inoculum on the Rumen Microorganisms of Lambs 498
J. O. Tucker, M. W. Glenn, and G. W. Roberstad

Muscular Dystrophy in Lambs as Related to the Tocopherol Levels in the Plasma and the Milk of Ewes and to Various Feeds..... 503
J. W. Safford, K. F. Swingle, and D. E. McRoberts

(Continued on page 339)

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(Contents continued from page 337)

Abortion and Pre- or Postnatal Death of Young Due to *Listeria Monocytogenes* III. Studies in Ruminants 510
M. L. Gray, C. Singh, and F. Thorp, Jr.

Plasma, Urine, and Bile Levels of 17-Hydroxycorticosteroids in Sheep and Cattle Following the Administration of Hydrocortisone 517
L. W. Holm and G. Firch

Isolation of a Pleuropneumonia-like Organism from Pigeons 521
W. J. Mathey, Jr., H. E. Adler, and P. J. Siddle

Study of Some Commercially Available Infectious Bronchitis Vaccines. I. Response of Chickens to Seven Infectious Bronchitis Vaccines 523
L. G. Raggi and R. A. Bankowski

Electrophoretic Analyses of Serum from Chickens Infected with Newcastle Disease Virus 531
M. A. Ginchereau and H. L. Chute

Antigenic Studies Using Ammonium Sulfate. IV. The Sedimentation Effect of Ammonium Sulfate on *Salmonella Gallinarum* 535
T. E. Williams and M. E. Harris

The Effect of Certain Antibiotics and Chemical Agents on Pleuropneumonia-like Organisms of Avian Origin 538
R. Yamamoto and H. E. Adler

Antigenic Studies on the Psittacosis-Lymphogranuloma Venereum Group of Viruses III. Detection of Ornithosis Hypersensitivity in Experimentally Infected Chickens 543
A. A. Benedict, E. O'Brien, and C. McFarland

Lysogeny in *Pasteurella Multocida* 547
C. Kirchner and A. Eisenstark

Survival Time of *Mycobacterium Paratuberculosis* 549
A. B. Larsen, R. S. Merkal, and T. H. Vardaman

A Simple Device for Carrying Multiple Pieces of Tissue Through Reagents from the Fixative into the Paraffin Block 552
J. G. Bowne and R. Getty

Actinobacillosis. I. An Evaluation of Cultural Characteristics of Selected Strains of *Actinobacillus Lignieresi* 555
M. Ristic, M. Herzberg, D. A. Sanders, and J. W. Williams

Serological Cross-Agglutination Reactions Between *Leptospira Pomona* and Leptospira Icterohaemorrhagiae, AB 563
E. V. Morse and V. Allen

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VOL. XVII JULY, 1956 No. 64

The Longitudinal Vertebral Venous Sinuses of the Dog
I. Anatomy

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Ames, Iowa

SINCE Batson's[2] initial report in 1940, study of the venous sinuses* of the spinal canal has supplied answers to many perplexing questions of metastasis and collateral circulation in clinical medicine in man. Part I of this research was undertaken to record the anatomy of this system of veins in the dog and to compare this anatomical pattern with that reported in man. Part II[21] is concerned with the functional aspects of these and related veins.

LITERATURE CITED

As early as the sixteenth century, medical literature recorded the existence of venous plexuses along the vertebral column of man. Harris[11] gave the most complete bibliographical and historical sketch of their early description. More recent anatomical accounts are numerous.[9-11, 25, 29] The embryological development of these venous channels of the body axis was also discussed.[1, 25, 28, 29]

In contrast to the literature concerning man, very little was found in veterinary writings covering this subject. Dräger[7] stated that Breschet, in 1836, had described the veins of the spinal canal of whales. Owen[17] described these also. Franklin[8] referred to these veins in whales, seals, beaver, and other diving mammals. Veterinary anatomy textbooks[4, 6, 25, 26] acknowledge the presence of the longitudinal vertebral sinuses in only a general way. Ellenberger and Baum[6] briefly discussed these veins for the dog. Their description was extracted from the report by Dräger[7] which was based on the dissection of these vessels in 8 dogs.

MATERIALS AND METHODS

The longitudinal vertebral sinuses and their connections with veins outside the spinal canal were dissected in 15 canine cadavers of mixed breeding. In this group, 7 were females and 8 were males; the age extremes ranged from 6 weeks to more than 10 years. Thirty-two additional cadavers were dissected, in part, to gain further information concerning the emissaries and tributaries of the sinuses. In all, 47 dogs were dissected.

All cadavers were embalmed. The arteries were injected with an aqueous suspension of red lead and starch. Special venous injections of a blue-colorcd latex suspension were used in 2 cadavers.

FINDINGS

Anatomy of the Longitudinal Vertebral Sinuses of the Dog.—Upon removal of the arches of the vertebrae and the spinal cord (fig. 1), the longitudinal vertebral sinuses were seen lying in the epidural space along the entire length of the floor of the vertebral canal. The posterior course begun by the ventral occipital sinuses from the postero-lateral wall of the cranial cavity (fig. 2) was continued without interruption by the right and left vertebral sinuses within the spinal canal. At its origin at the foramen magnum and throughout the atlas, each vertebral sinus appeared ampullated and larger than at any other point in its course. Within the third cervical vertebra the sinuses became slightly smaller, but they remained uniformly large throughout the cervical region. A definite decrease in caliber of the longitudinal vertebral sinuses was evident at the thoracic inlet. After this reduction, however, their diameters remained quite constant throughout the thoracic and most of the lumbar regions. At the level of the fifth lumbar segment, a marked reduction in the caliber of the sinuses began which was progressive from one segment to the next. They were reduced to two fine threads at the fifth or sixth coccygeal segment where they could

From a thesis submitted in 1953 in partial fulfillment of the requirements for the degree of master of science in veterinary medicine, Iowa State College, Ames. The author is now in the Department of Veterinary Anatomy, College of Veterinary Medicine, State College of Washington, Pullman.

* The term for these vessels in the 1955 revision of the "Nomina Anatomica" is plexus venosi vertebrales interni.

no longer be traced in the dorsal tail musculature beyond the termination of the vertebral canal.

Within the atlas and between the atlas and the axis, the large sinuses lay against the sides of the vertebral arches so that the spinal cord passed between the two in contact with the dorsal surface of the dens of the axis. Because of this lateral displacement, the first cervical nerve invariably penetrated the sinus of either side to emerge from the spinal canal. Posterior to the axis, the sinuses were more medially located and lay upon the floor of the vertebral canal against the dorsal longitudinal ligament.

The longitudinal vertebral sinuses were characterized by their segmentally arranged arcs, the right and left convexities of which approached each other in the middle of each vertebral segment (fig. 1). The curvature of these two arcades varied, being greatest in the lumbar and sacral regions and the least arched in the coccygeal and anterior half of the thoracic regions. At each intervertebral foramen, where one arch joined the next in the arcade, intervertebral veins (fig. 3, 4) connected the sinus of the side with veins outside the vertebral canal. Small radicles from the plexus of veins along the spinal cord followed the dorsal and ventral roots of the spinal nerves and joined the intervertebral veins at each foramen.

In the middle of each vertebra, the convexities of the right and left sinuses were connected across the midline beneath the dorsal longitudinal ligament. These deep transverse anastomoses passed beneath the ligament only, or in a channel through the bone beneath the ligament (fig. 4). Basivertebral veins made connections with these deep anastomoses. By passing through canals within the vertebral bodies, these basivertebral veins emerged through venous foramina on the ventral side of the bodies of the vertebrae and joined extravertebral veins.

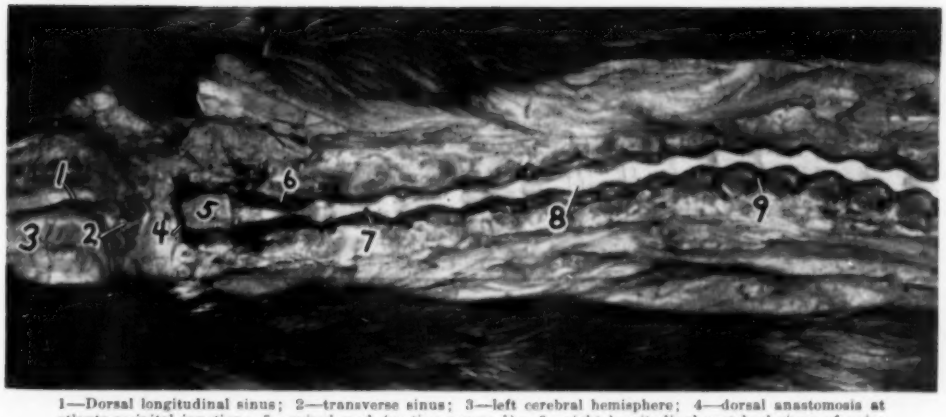
In addition to the deep transverse anastomoses, there were also superficial connections between the convexities of the two sinuses above the dorsal longitudinal ligament (fig. 2). These were less constant in location and much fewer in number. Besides the interoccipital sinus at the level of the foramen magnum, a superficial ventral anastomosis was usually present in the axis. Infrequently, anastomoses of this type were seen in the other cervical segments. No superficial ventral anastomoses occurred with regularity in the other regions.

Dorsal anastomoses between the two longitudinally coursing sinuses were found only in the anterior part of the cervical region (fig. 2). These passed dorsally across the vertebral canal beneath the anterior edge of the arch of the atlas and between the atlas and the axis. Occasionally, a dorsal anastomosis was also found between the second and third cervical vertebrae. What appeared to be dorsal anastomoses in other segments invariably proved to be two epaxial veins, right and left, which entered the vertebral canal by penetrating the ligamentum flavum in the interarch space. These did not connect with each other across the midline within the epidural space.

Emissaries and Tributaries of the Longitudinal Vertebral Sinuses.—These sinuses were seen to be the direct continuations of the ventral occipital sinuses posterior to the foramen magnum (fig. 2). The ventral occiptal sinus on each side of the basilar part of the occiptal bone was formed by the confluence of the condyloid sinus and the basilar sinus. Each condyloid sinus was connected anteriorally through the condyloid canal to the posterior division of the transverse sinus of its side, while each basilar sinus was continued anteriorally by the cavernous sinus.

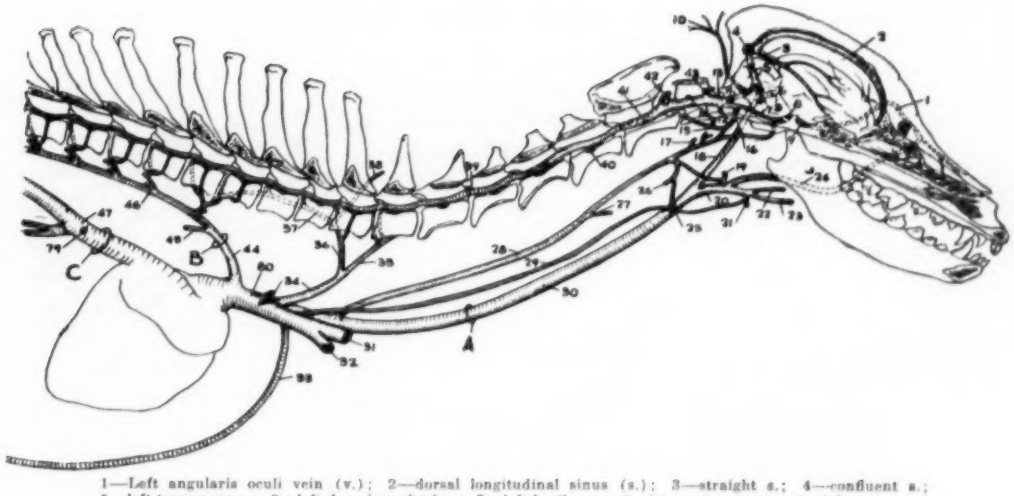
Intervertebral Connections in the Cervical Region.—At every intervertebral foramen, the sinus of each side made connections by intervertebral veins with veins lying outside the spinal canal (fig. 2). The first intervertebral vein draining the longitudinal vertebral sinuses emerged from the intervertebral foramen of the atlas and joined the occipital vein. The occipital vein coursed ventral to the wing of the atlas by way of the alar notch. Here it joined the condyloid and ventral cerebral veins to form the internal jugular vein.

The second to the eighth intervertebral veins of the cervical region drained into the vertebral vein as it coursed through the transverse canal. Because of the absence of a transverse canal in the seventh cervical vertebra, the vertebral vein coursed ventral to the transverse process. The vertebral vein, at its anterior extremity, continued through the transverse foramen of the atlas to join the occipital vein by a large branch beneath the wing of the atlas. The second intervertebral vein, that between the atlas



1—Dorsal longitudinal sinus; 2—transverse sinus; 3—left cerebral hemisphere; 4—dorsal anastomosis at atlanto-occipital junction; 5—spinal cord (section removed); 6—right longitudinal vertebral sinus of axis; 7—dorsal longitudinal ligament; 8—intervertebral disc; 9—third thoracic intervertebral vein.

Fig. 1—Calvarium and epaxial structures removed exposing the longitudinal vertebral sinuses (dorsal view).



1—Left angularis oculi vein (v.); 2—dorsal longitudinal sinus (s.); 3—straight s.; 4—confluent s.; 5—left transverse s.; 6—left dorsal cerebral v.; 7—left basilar s.; 8—intercavernous s.; 9—left cavernous s.; 10—emissary v.; 11—left condyloid s.; 12—left condyloid v.; 13—left ventral occipital s.; 14—inter-occipital s.; 15—left occipital v.; 16—left ventral cerebral v.; 17—pharyngeal plexus; 18—left internal maxillary v.; 19—left anterior laryngeal v.; 20—left external maxillary v.; 21—hyoid arch v.; 22—left lingual v.; 23—left sublingual v.; 24—left facial v.; 25—posterior laryngeal arc v.; 26—left anterior thyroid v.; 27—left posterior thyroid v.; 28—left internal jugular v.; 29—thyroidima v.; 30—left external jugular v.; 31—right external jugular v. (cut); 32—right axillary v.; 33—left internal thoracic v.; 34—right and left costocervical-vertebral trunks; 35—left vertebral v.; 36—left costocervical v.; 37—left supreme intercostal v.; 38—left deep cervical v.; 39—left longitudinal vertebral s.; 40—deep ventral transverse anastomosis; 41—superficial ventral transverse anastomosis; 42—dorsal anastomosis; 43—inter-vertebral branch of occipital v.; 44—azygos v.; 45—broncho-oesophageal v.; 46—right and left sixth intercostal v.; 47—posterior vena cava; 79—right phrenic v.; 80—anterior vena cava.

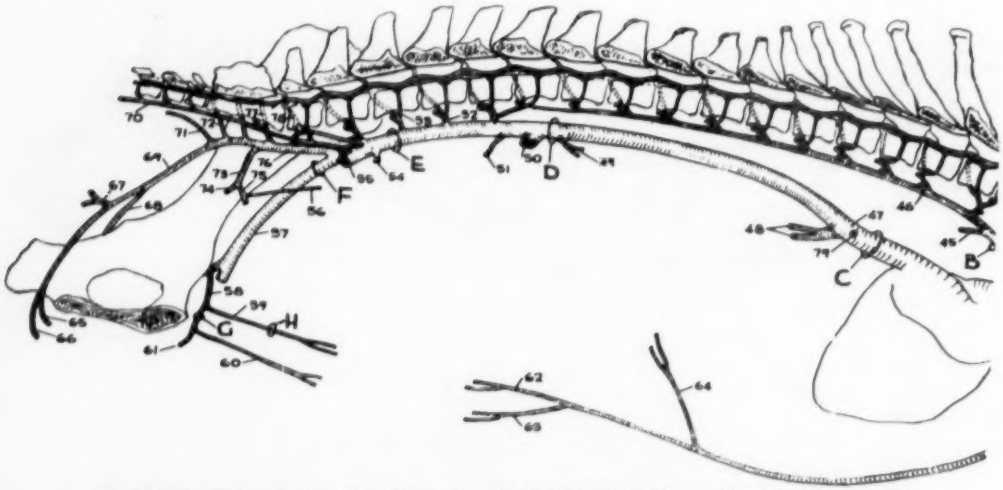
Fig. 2—Plan of the systemic veins of the head, neck, and anterior thoracic region. (Capital letters indicate points at which veins were ligated in experiments to be described in Part II.)

and the axis, was invariably single, while the other six of the cervical region posterior to it were double. The anterior and posterior veins of each pair passed through the intervertebral foramina by way of the posterior and anterior notches of the contiguous vertebrae. The vertebral vein terminated by draining into the costocervical-vertebral trunk vein. This, in turn, emptied into either the anterior vena cava or the brachiocephalic trunk.

Intervertebral Connections in the Thoracic Region.—The costocervical vein on each side was formed by the confluence of the deep cervical and the supreme intercostal veins. Without exception, the first intercostal vein and its unpaired intervertebral vein joined the deep cervical vein high in the first intercostal space (fig. 2, 3). Posterior to the first space, the supreme intercostal vein, coursing on each side dorsal to the necks of the ribs, received the second and third intervertebral and intercostal veins on the right side. On the left side, the fourth vein also joined the supreme intercostal vein in almost half of the specimens. Posteriorally, the supreme intercostal vein joined the first intercostal branch of the azygos vein.

The azygos vein received the right and left intercostal veins of the remaining thoracic vertebrae. Single intervertebral branches connected the longitudinal vertebral sinuses to each of these intercostal veins at the highest point in the intercostal spaces of each side. A vein of small caliber was always present which joined the azygos to the first lumbar vein. This posterior extension of the azygos vein coursed along the midline beneath the bodies of the last thoracic and first lumbar vertebrae.

Intervertebral Connections in the Lumbar Region.—Each intervertebral vein of this region emerged from its foramen as a double vessel which quickly united to form a single lumbar vein on each side (fig. 3, 4). Right and left lumbar veins then passed downward and forward on either side of the bodies of the vertebrae and emptied into the azygos, the posterior vena cava, the common iliac, the iliolumbar, or the middle sacral veins. The right and left veins of all but the first and last lumbar segments regularly joined each other be-



45—Broncho-oesophageal vein (v.); 46—right and left sixth intercostal v.; 47—posterior vena cava; 48—hepatic v.; 49—right phrenico-abdominal v.; 50—right renal v.; 51—right (internal) spermatic v.; 52—fourth lumbar v.; 53—basivertebral v.; 54—right deep circumflex iliac v.; 55—right common iliac v. (cut); 56—left ureteral v.; 57—left external iliac v.; 58—left pudendo-epigastric trunk; 59—left posterior deep epigastric v.; 60—left posterior superficial epigastric v.; 61—scrotal branches of left external pudendal v.; 62—left anterior deep epigastric v.; 63—left anterior superficial epigastric v.; 64—left musculophrenic v.; 65—left dorsal v. of penis; 66—left deep v. of penis; 67—left perineal-posterior hemorrhoidal trunk; 68—left posterior gluteal v.; 69—left internal pudendal v.; 70—middle sacral v.; 71—left lateral superficial coccygeal v.; 72—left third sacral intervertebral v.; 73—left urogenital v.; 74—left urethral v.; 75—left prostatic-posterior vesical trunk; 76—left internal iliac v.; 77—first sacral v. to anterior gluteal v.; 78—intervertebral branch to iliolumbar and seventh lumbar v.; 79—right phrenic v.

Fig. 3—Plan of the systemic veins of the trunk of the male dog. (Capital letters indicate points at which veins were ligated in experiments to be described in Part II.)

neath the vertebral bodies to form a common trunk which then emptied into the extravertebral veins mentioned.

The first lumbar segment was always drained by a right and a left lumbar vein which emptied separately into the posterior extension of the azygos vein along the midline. Occasionally the second lumbar vein was a tributary of the azygos, but as a rule the second was drained by an unpaired vessel into the third lumbar vein. There was usually a definite venous connection between the azygos and the posterior caval systems. This was seen in 13 of the 15 dogs as a small vein which united the first and second lumbar veins ventral to the vertebrae along the midline. The third lumbar vein drained as a single trunk into the posterior vena cava after receiving its right and left intervertebral veins and the tributary from the second vertebra which was mentioned above. In two instances, the third joined the fourth lumbar vein instead of the posterior vena cava. The sixth lumbar vein was an unpaired tributary of the posterior vena cava in nine instances, the right common iliac vein in two, and the left in four instances. The chief intervertebral drainage of the seventh lumbar segment was by way of the large right and left iliolumbar veins in every specimen. In 9 dogs, the intervertebral branch of the iliolumbar vein of each side was joined by a small branch from the middle sacral vein and in 6 by a small branch from the common iliac veins.

Intervertebral Connections in the Sacral and Coccygeal Regions.—The longitudinal vertebral sinuses of the sacral region were drained by way of the anterior gluteal or the internal pudendal veins (fig. 3) into the right and left internal iliac veins. The first sacral vertebra of all specimens was drained through its ventral sacral foramen on each side by an intervertebral branch of the anterior gluteal vein. In the majority of the dogs, a second intervertebral branch of each anterior gluteal vein passed through the second ventral sacral foramen but, not infrequently, the second sacral segment was drained by a separate tributary of each internal pudendal vein. The sinuses of the third sacral segment were always drained by a separate branch of the internal pudendal vein on each side.

The sinuses in the coccygeal region drained almost exclusively into the middle coccygeal vein, the posterior continuation of the middle sacral vein. In 16 of 42 dogs examined, the middle sacral vein sent tributaries to the right and to the left common iliac veins. In 17 specimens, it entered only

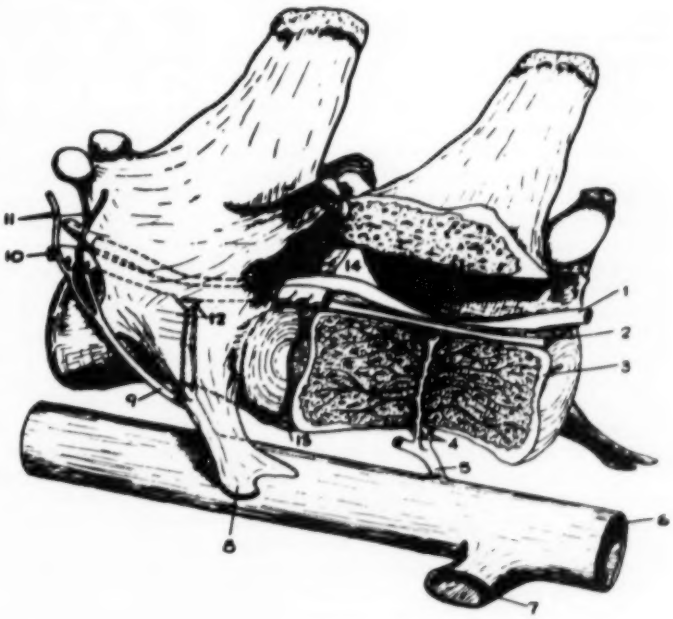


Fig. 4—Plan of the emissaries of the longitudinal vertebral sinuses in the third and fourth lumbar segments. (The third lumbar vertebra is cut sagittally to the right of the midline.)

1 — Left longitudinal vertebral sinus; 2—dorsal longitudinal ligament; 3—body of third lumbar vertebra (cut sagittally); 4—paired basivertebral veins; 5 — third lumbar vein (v.). (The second lumbar v. usually drains into the third but is not shown.); 6—posterior vena cava; 7—right renal v.; 8—transverse process of fourth lumbar vertebra; 9 — right fourth lumbar intervertebral v.; 10 — right longitudinal vertebral sinus; 11—Epaxial branches; 12—deep ventral transverse anastomosis; 13—intervertebral disc; 14—left third lumbar intervertebral foramen.

the right vein and in nine, only the left common iliac vein. All the intervertebral branches of the coccygeal region, posterior to the first, drained into the middle coccygeal vein. On one side only, the first coccygeal segment was twice drained by a separate branch from the internal pudendal and twice from the lateral superficial coccygeal vein. In all other instances, the middle coccygeal vein received the right and the left intervertebral veins from the coccygeal segments.

Basivertebral Veins.—Basivertebral veins (fig. 3, 4) were found in all regions of the vertebral column. They passed through channels within the bodies of the vertebrae and connected the deep transverse anastomoses of the longitudinal vertebral sinuses with the extravertebral veins of the region. They emerged through variable foramina which usually lay just to the right, or to the left, of the midline on the ventral side of the vertebral bodies. Only in the lumbar region were both right and left foramina found with some degree of regularity. When two foramina were present in any segment, the foramen of one side was often much larger than the other in the same vertebra. Whether one or more foramina were present on the ventral side of the vertebra, the basivertebral veins passing through the body of the vertebra always joined both the right and the left longitudinal vertebral sinuses at the deep transverse anastomosis.

In the cervical region, branches from the vertebral vein in the transverse canal of each side passed downward and backward through the longus colli muscle to connect with the basivertebral veins. Other inconstant veins drained the arches and bodies of the vertebrae of the cervical region. They emptied into these sinuses and into the vertebral veins. In some anterior segments of the thoracic region, no basivertebral veins were evident. Most frequently, however, single basivertebral veins were seen entering the intercostal veins in an irregular manner. In the lumbar region, the lumbar veins usually received right and left basivertebral veins through paired foramina on the ventral side of the vertebral bodies. The sacral and coccygeal vertebrae most frequently showed no basivertebral veins, but when they were present they emerged from the vertebrae as a single vessel to enter into the middle sacral vein.

Interarch Connections.—The dorsal anastomoses, described earlier as coursing beneath the anterior edge of the arch of the atlas and between the atlas and the axis, received affluents through the interarch spaces from the musculature of the poll. In the next three or four cervical segments, epaxial veins passed through the ligamenta flava of the interarch spaces to the right and to the left of the midline. Upon entering the epidural space, they coursed ventrally along the arches of each side to enter these sinuses. In the remainder of the cervical and anterior thoracic segments, identical veins passed through the epidural space in a similar fashion. Instead of being tributaries of the sinuses, however, they joined the intervertebral veins at their emergence from the spinal canal. Veins from the epaxial region of the posterior half of the body joined the intervertebral veins outside the vertebral canal without first gaining entrance into the epidural space through the interarch spaces.

Valves.—No valves were found within the cavities of the sinuses themselves. The intervertebral veins connecting the vertebral veins with the cervical sinuses also had no functional valves.

In the other regions, however, one or more valves were found in the veins which connected the intervertebral vessels with their major extravertebral vein. Thus, each lumbar vein possessed a valve at its entrance into the posterior vena cava. The free edges of these valves were directed toward the major extravertebral vein and away from the sinuses.

DISCUSSION

The anatomy of the longitudinal vertebral sinuses of the dog as described by Dräger[7] and summarized by Ellenberger and Baum[8] was confirmed, for the most part, by this work. It presents some striking differences from that reported in man.[9-11,13,19] Compared to man, the vertebral sinuses of the dog showed a marked reduction in their plexiform arrangement. Only the ventral, longitudinally coursing pair of vessels was present, there being a complete absence of the dorsal pair of veins described in man. The venous rings which circle the lumen of each vertebral segment in man were suggested only in the cervical region in the dog. Here they were represented by the ventral deep transverse anastomoses and the dorsal transverse anastomoses in the anterior cervical segments.

In the procedure outlined by Brook[5] for the withdrawal of cerebrospinal fluid at the

foramen magnum of the dog, the dorsal transverse anastomosis which courses transversely just anterior to the arch of the atlas would appear liable to trauma. Its caliber is relatively small and would probably result only in contamination of the fluid sample with blood. Were the aspirating needle directed to either side of the midline at this point, however, these sinuses themselves might be penetrated. They are very large at the atlanto-occipital junction and lie against the sides of the vertebral canal at this level. Similarly, in administering an epidural anesthetic at the lumbosacral junction in the dog, the possibility of injecting directly into the right or left vertebral sinus must be considered. Such an occurrence was reported in man by Berens.[3]

The veins which drain the sinuses were seen to empty into the occipital and vertebral veins in the cervical region and into the costocervical and the azygos veins in the thoracic region.[7,8] The latter authors stated that the posterior vena cava received six lumbar veins; Bradley[4] said six or seven. Dräger mentioned seven lumbar veins but admitted that all did not empty into the posterior vena cava. In this series of dogs, the first lumbar vein was always a tributary of the azygos vein. Sometimes the second and third were also. The second usually drained into the third, however. The third, fourth, fifth, and usually the sixth, drained into the posterior vena cava. The sixth may be a tributary of the right or the left common iliac veins. The largest vein draining the seventh lumbar segment was always a branch of the iliolumbar vein at the lumbosacral junction. This branch was routinely joined by a small vein from the middle sacral or common iliac veins, and these always received the basivertebral veins from that segment.

In 1 of the dogs dissected, the right and the left common iliac veins did not unite to form the posterior vena cava until the second lumbar vertebra was reached. In this animal, each renal vein emptied into the origin of the posterior vena cava, and the usual caval lumbar veins then drained into the right common iliac vein. Gray[10] mentioned that this may also occur in man. In this series of dogs, the second lumbar vein, with but few exceptions, anastomosed directly with the azygos vein, thus uniting the caval and azygos systems. Miller[14] and Hickman *et al.*[12] cited anomalies in man and the dog, respectively, in which the posterior vena cava was continued anteriorally by the azygos vein.

The first and second sacral veins joined the anterior gluteal veins, although in almost half of the cadavers the second was a separate tributary of the internal pudendal. Dräger[7] made no mention of the third sacral vein, but it invariably drained into the internal pudendal vein.

According to Dräger, the drainage from the coccygeal region was via the middle sacral vein. This most frequently joined the left common iliac vein, according to Ellenberger and Baum.[5] Instead, in 33 of 42 dogs examined, this vessel was a tributary of the right or both the right and the left common iliac veins. No connection between the sinuses and the lateral superficial coccygeal vein was seen by Dräger.[7] In the present group, on one side only, the first coccygeal vein drained into the internal pudendal vein in 2 dogs and into the tail vein in 2 others. The remaining coccygeal veins emptied into the middle sacral vein via the middle coccygeal vein.

Summary and Conclusions

1) The anatomical pattern of the longitudinal vertebral sinuses of the dog was described from the detailed dissection of 15 canine cadavers. Thirty-two additional cadavers were examined in part to supplement the 15 basic dissections.

2) The longitudinal vertebral sinuses course along the floor of the epidural space throughout the length of the spinal canal of the dog. Beginning at the foramen magnum, as direct posterior continuations of the two ventral occipital sinuses, they are characterized by their segmentally arranged arcs, the right and the left convexities of which approach each other in the middle of each vertebral segment. At each intervertebral foramen, where one arch joins the next in the arcade, intervertebral veins connect the sinuses of that side with surrounding veins outside the vertebral canal. In the middle of each segment, the convexities of the right and the left sinuses are united across the midline beneath the dorsal longitudinal ligament by a deep transverse anastomosis. Basivertebral veins within the vertebrae make connection with these deep anastomoses, and, by emerging through foramina on the ventral surface of the vertebral bodies, they drain into the extravertebral veins of that region. Superficial ventral anastomoses are similarly located to the deep anastomoses, but they

unite the convexities of the two sinuses above the dorsal longitudinal ligament. They are much fewer in number and are largely confined to the anterior cervical segments. Dorsal anastomoses are found which unite the two sinuses anterior and posterior to the arch of the atlas. Here the sinuses are very large and located lateral to the spinal cord, being pierced by the first cervical nerve. Within the axis they become more ventrally placed and somewhat reduced in size. They remain quite large throughout the cervical region, however, becoming of lesser caliber at the level of the thoracic inlet. After this they remain quite constant in diameter until the fifth lumbar segment is reached. Here a marked reduction in caliber begins from one segment to the next until they are lost as two fine threads in the epaxial musculature of the fifth or sixth coccygeal segment.

3) The vertebral sinuses of the dog show a marked reduction in their plexiform arrangement as compared to man. Only the ventral, longitudinally coursing pair of vessels is present in the dog. The complete venous rings which circle the lumen of each vertebral segment of man are present in only the first two or three cervical segments of the dog.

4) The large size of the longitudinal vertebral sinuses of the dog just posterior to the foramen magnum would make them liable to injury were the needle directed to either side of the midline while withdrawing a sample of cerebrospinal fluid at the atlanto-occipital junction. In giving an epidural anesthetic at the lumbosacral junction of the dog, the possibility of injecting directly into the right or the left vertebral sinus must be considered.

5) The anatomical pattern of the venous connections with the longitudinal vertebral sinuses of the dog was also described. These connections include the intervertebral, the basivertebral, and the interarch veins.

6) No valves were found within the lumens of the sinuses themselves. In all but the cervical region, valves were regularly found in the veins which connected the intervertebral vessels with their major extravertebral vein. The free edges of these valves were directed toward the extravertebral vein and away from the sinuses.

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The Longitudinal Vertebral Venous Sinuses of the Dog
II. Functional Aspects

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THE ANATOMY of the longitudinal vertebral sinuses of the dog was described in Part I.[47] Part II of this study was undertaken to demonstrate the pattern of venous drainage by the longitudinal vertebral sinuses of the dog and to investigate the clinical significance of these vessels.

LITERATURE CITED

The veins of the vertebral canal were well described and understood over 100 years ago.[14] Credit, however, must be given to Batson[1, 2] for his rediscovery of this system of veins early in the last decade and for demonstrating its clinical significance in man. He concluded from venographic studies of human cadavers and living monkeys that, since the vertebral vein system parallels, connects with, and provides bypasses for the portal, the pulmonary, and the caval systems of veins, it could provide, in itself, a pathway for the spread of disease between remote organs. Therefore, he proposed that the vertebral system, because of its anatomical, physiological, and pathological importance, be added as a fourth system to the recognized system of veins.

Since Batson's report, similar injection experiments and findings were reported by others.[12, 22, 28] Some advocated the adoption of the vertebral veins as a fourth venous system.[6, 22] Numerous references in medical literature involve this route in a wide variety of metastatic and pathological conditions and indicate the prominent place to which the vertebral vessels have risen in clinical medicine in man.[4, 7, 9, 25, 29, 42] Since the revelation by Batson of the adequacy of the collateral circulation of the body's axis, many large venous trunks are purposefully ligated.[14, 26, 27, 63] Inferior vena caval ligation was previously done only as a result of accidental injury to this vein during the course of abdominal operations. Walters and Priestly[66] considered such a ligation as a last resort measure. Today, however, the inferior vena cava is totally ligated below the level of the renal veins as a life-saving measure for conditions such as septic or bland embolism from the limbs or pelvis.[10, 23, 35, 48, 66] More than 100 cases of caval ligation as a palliative measure in cardiac decompensation are reported.[21]

The physiological and pathological significance of the spinal venous sinuses in our domesticated animals is not well described in veterinary literature. Medical research, directly or indirectly, does involve these vessels in a number of animal species, however. Harris[14] stated that Cruveilhier, in 1834, had ligated both external jugular veins in a dog without ill effect, the circulation being maintained by the veins of the spine. Kershner, Hooton, and Shearer[24] ligated the posterior vennæ cavæ of 46 dogs in an attempt to produce experimental portal hypertension. If the ligations were made posterior to the liver, they were fairly well tolerated because of the existence in the dog of collateral venous pathways. Wittenberger and Huggins[65] reported that sudden obstruction of the posterior vena cava above the renal level was fatal in dogs, whereas ligation is well tolerated below the renal veins. Pfaff[37] reported similar findings. Lespinasse[30] reviewed this subject and said that Polkey's 5 dogs died when the posterior vena cava was ligated anterior to the renal veins. He then described his own experiments on 1 dog and on a number of rats, rabbits, cats, and monkeys. By ligating the posterior vena cava behind the renal veins two weeks before ligating anterior to them, the anastomotic channels along the spine were given time to develop and the percentage of survival was increased. Lew[27] reported that the mortality rate of rats increased with the age of the animal subjected to caval ligation. Moore and Lawrence[31] injected carcinoma suspensions into the femoral veins of rabbits that previously had had the posterior vena cava ligated.

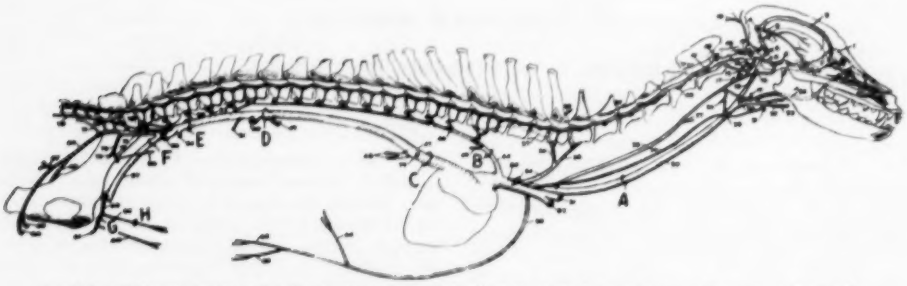
MATERIALS AND METHODS

A total of 22 mongrel dogs was subjected to a variety of experimental procedures in this work. In 16 of these dogs, a number of large venous trunks were ligated at eight different sites (fig. 1). The ligatures used were most often linen; pentobarbital sodium solution was the anesthetic. The intra-abdominal vessels were approached by a midline, transperitoneal route, while the thoracic veins were reached by entering the pleural cavity according to the method suggested by Markowitz.[26]

Forty-five venographs of 17 living dogs were completed in this study, 11 of which are photographically reproduced in this paper. These venographs were utilized to indicate the course of venous return in normal, anesthetized dogs as well as in those altered by ligations or elevated body pressure. Multiple points of injection of the radiopaque material were often used, and the radiographic results were confirmed by detailed dissection of the embalmed animals.

From a thesis submitted in 1953 in partial fulfillment of the requirements for the degree of master of science in veterinary medicine, Iowa State College, Ames. The author is now in the Department of Veterinary Anatomy, College of Veterinary Medicine, State College of Washington, Pullman.

The diodrast ℞ used in this work was supplied through the courtesy of Winthrop-Stearns Inc., New York, N. Y.



A—External jugular vein (v.); B—azygos v.; C—intrathoracic posterior vena cava; D—prerenal posterior vena cava; E—postrenal posterior vena cava; F—external iliac v.; G—external pudendal v.; H—posterior deep epigastric v.

Fig. 1—Plan of the systemic veins of the head, neck, and trunk of the male dog. Capital letters indicate points at which veins were ligated. (For legend, see fig. 2 and 3, Part I, pp. 343, 344.)

Diodrast* was the contrast medium used in the venographic studies. This was injected by means of a glass syringe through a blunted needle which had been ligated into the exposed vein of choice. In each case, the circulation time was first determined by injecting 0.5 cc. of ether in 4.5 cc. of normal saline solution into the peripheral vein chosen. The time required after beginning the injection to detect ether upon the subject's breath was recorded as the "ether time" and served to indicate the speed of venous return from that vein to the heart and lungs. From 8 to 10 cc. of diodrast (for a dog the size of a Fox Terrier) was then injected into the peripheral vein chosen and a radiographic exposure completed, usually within the ether time interval.

A general electric model D3 mobile shockproof x-ray unit was used for the entire work. Satisfactory venographs were obtained by using kodak blue brand medical x-ray film in an aluminum cassette with Patterson par-speed intensifying screens. On certain individuals, a wafer grid or a cone, or both, were used. The technique varied with the size of the subject, but a typical setting for a dog that measured 4 inches through the abdomen was 26-inch film target distance, 74 kv. p., 15 ma. and 3 ma. per second of exposure. Development and fixing were done according to the standard time-temperature method.

* Diodrast concentrated aqueous solution 70 per cent® (Winthrop-Stearns, Inc.)

INVESTIGATION AND FINDINGS

INVESTIGATION 1

Objective.—The purpose was to determine the effect upon the living animal of ligating the posterior vena cava posterior to the renal veins and to demonstrate the place of the longitudinal vertebral sinuses as a collateral route of the body axis.

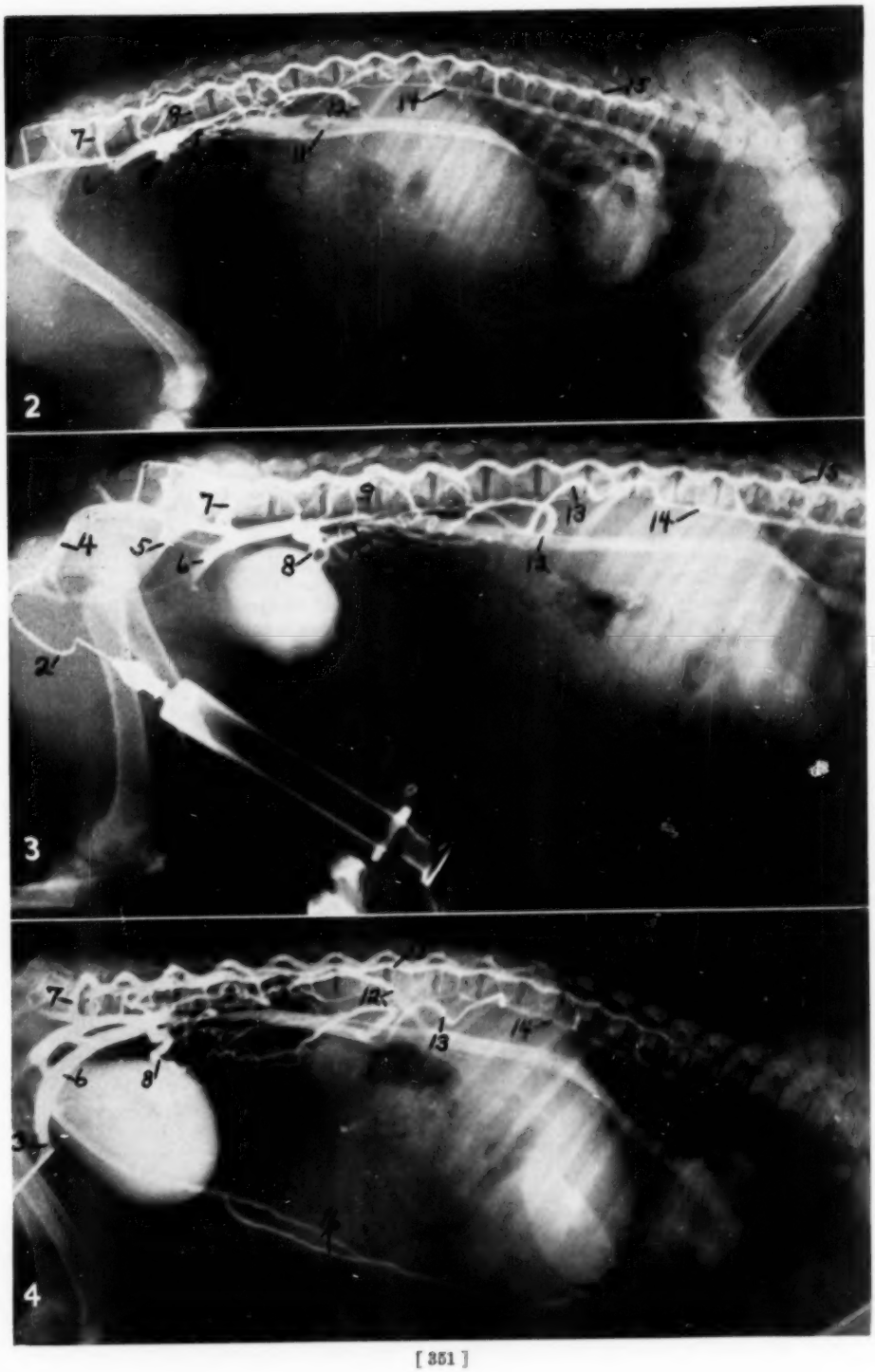
Method.—In 4 dogs, the posterior vena cava was ligated just anterior to the deep circumflex iliac veins. Dog 1, a 3.5-year-old male, was observed for six months subsequent to ligation. Three venographs of dog 2, a 6-month-old female, were made after 16 days while injecting diodrast into the lateral superficial coccygeal (tail) and femoral veins. Six weeks after ligation, similar injections for venography were made into the dorsal vein of the penis, the femoral and the tail veins in dog 3, a 3-month-old male. A tail vein venograph of dog 4, a 13-week-old male, was made 45 minutes after ligation. Two weeks later, additional exposures were made while injecting into the tail and the saphenous veins.

Results.—Immediately after ligation, a cyanosis of the hindquarters developed

Legends for Illustrations on Opposite Page

Fig. 2, 3, 4—Successive venographs of dog 3 made six weeks after posterior vena cava ligation. Injections of diodrast® into different veins were required to visualize the entire venous return of the trunk. Notice the progressive accumulation of diodrast in the urinary and gall bladders.

1—Injection into right tail vein (v.); 2—injection into right dorsal v. of penis; 3—injection into left femoral v.; 4—internal pudendal v.; 5—urogenital and posterior ureteral veins; 6—external iliac v.; 7—iliolumbar v.; 8—deep circumflex iliac v.; 9—basivertebral v.; 10—point of caval ligation; 11—entrance of renal v.; 12—phrenicoabdominal v.; 13—anastomosis between 8 and the twelfth intercostal v.; 14—azygos v.; 15—longitudinal vertebral sinus; 16—anastomosis between posterior and anterior deep epigastric veins.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which disappeared within 24 hours in all animals. Only in dog 1 was there a slight edema of the scrotum and hindpaws after exercise during the first week. None showed discomfort at any time and all appeared to be normal, active dogs.

Venographs and dissection of the dogs revealed that other venous channels existed which could effectively circumvent the occluded posterior vena cava. Most of these appeared to result from the enlargement of previously existing venous channels and anastomoses. Among them, the longitudinal vertebral sinuses were most consistently seen to be enlarged, especially in the lumbar region, and the most functionally active, radiographically. Venographs of dog 4, taken before and two weeks after ligation, show this enlargement well. Routes of venous return that were typical for the group are shown (fig. 2, 3, and 4). Multiple injections into several different veins were necessary to demonstrate all these routes. The intervertebral and basivertebral veins, posterior to the caval ligature, were tributaries of the longitudinal vertebral sinuses, while those anterior to it were its emissaries. These drained from the sinuses into the posterior vena cava anterior to the ligature and into the azygos vein. Enlarged and direct anastomoses between the deep circumflex iliac and the phrenicoabdominal veins were the rule. Branches from these anastomoses often joined intervertebral, basivertebral, and intercostal veins anterior to the ligature. Either the posterior superficial or posterior deep epigastric veins drained directly into its anterior counterpart. Although clinically normal, dogs 2 and 4 developed an extensive organizing thrombus posterior to the caval ligature. In these 2 animals, depending on the sex, either the posterior ureteral or the uterine veins were much enlarged. Even in the other 2 dogs, however, these vessels were seen radiographically to drain anteriorally. Only in dog 1 did the posterior hemorrhoidal veins appear enlarged. In none of the specimens was their drainage into the portal system proved radiographically.

INVESTIGATION 2

Objective.—The purpose was to determine the effect upon the subject, and upon the drainage of the longitudinal vertebral sinuses, of ligating both the posterior vena cava and the azygos vein.

Method.—In dog 5, a 6-week-old female, the posterior vena cava was ligated posterior to the renal veins. After 75 days the azygos vein was ligated, and two venographs were made. In dog 6, a 4.5-month-old male, the posterior vena cava was ligated as above, followed in 60 days by azygos vein ligation. Six days later, five venographs were made.

Results.—Neither dog showed untoward effects from either ligation. Venous drainage under these conditions is illustrated (fig. 5). The venous sinuses were well injected. Also, the deep epigastric veins were made opaque. However, only the posterior part of the azygos vein could be demonstrated radiographically. In this series of venographs, it was evident that diodrast had progressed much farther anteriorally in the longitudinal vertebral sinuses than it had in the experiments (fig. 2) in which the posterior vena cava alone was ligated.

INVESTIGATION 3

Objective.—The purpose was to determine the effect upon the dog of ligating the posterior vena cava anterior to the renal veins and to demonstrate the part played by the longitudinal vertebral sinuses in draining blood from the kidneys under such conditions.

Method.—The posterior vena cava was ligated just anterior to the renal veins of dog 7, a 2.5-year-old male, and dog 8, a 6-month-old female. After five weeks, two venographs of dog 7 were made. Two venographs were taken of dog 8 after 18 days, at which time the posterior vena cava was ligated again, this time posterior to the renal veins. Two weeks later, two additional venographs were made.

Results.—Upon ligation of the posterior vena cava, the kidneys of both dogs at once became turgid, and the skin of the posterior half of the body turned lavender. Urine removed by catheter from dog 7 was grossly bloody after 20 minutes. Within 24 hours, the urine was macroscopically free of blood and normal in volume. Dog 8 did not experience this initial severe reaction, and both dogs showed no untoward reaction after the first day.

The posterior vena cava of dog 7 showed almost complete occlusion by a thrombus from the point of ligation to the origin of the common iliac veins. Kidney drainage appeared to be largely by way of a maze of thin-walled perirenal veins which anastomosed freely with the phrenicoabdominal veins. These, in turn, drained into the

posterior vena cava anterior to the ligature and into the last two intercostal veins. Many of the small veins along the thrombosed posterior vena cava drained into the longitudinal vertebral sinuses via the lumbar veins. There was also an extensive subcutaneous and deep anastomosis of the anterior gluteal, deep circumflex iliac and phrenicoabdominal veins with the intercostal, anterior, and posterior epigastric and the external thoracic veins.

Dog 8 showed no thrombus formation. The venous drainage, 18 days after caval ligation anterior to the renal veins, is shown (fig. 6). Figure 7, taken two weeks after the second ligation, shows the isolated caval segment into which renal blood must flow. Exit for blood from this segment was by way of the enlarged lumbar branches of the longitudinal vertebral sinuses and the azygos vein. Grossly and microscopically, the kidneys of both dogs appeared normal, although large venous channels were prominent throughout the histological sections from dog 7.

INVESTIGATION 4

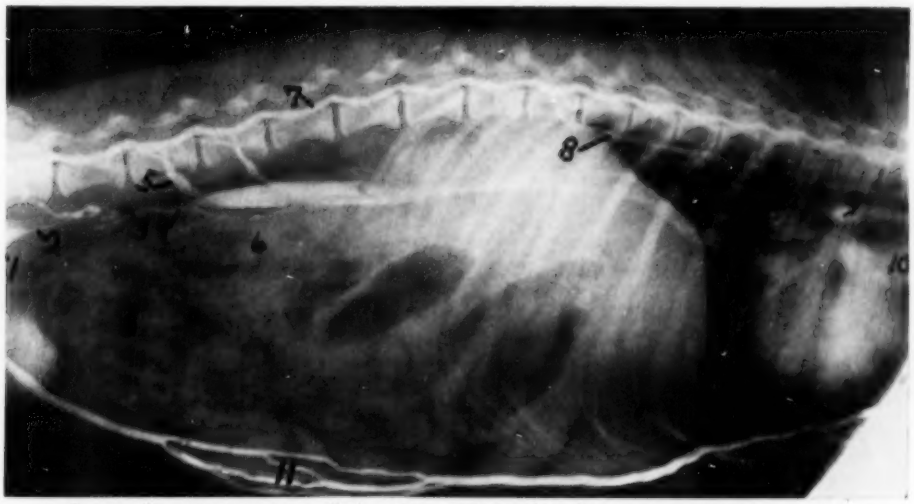
Objective.—The purpose was to determine whether there are enough collateral circuits available to maintain life when both posterior caval and hepatic blood are denied direct access to the heart.

Method.—In dog 9, a 2.5-year-old female, and in dog 10, a 3-year-old male, the midpoint of the intrathoracic posterior vena cava was ligated. The right phrenic nerve was spared. Respiration was maintained by a forced air respirator and tracheal intubation. In dog 10, the posterior vena cava had been ligated posterior to the kidneys for 16 days before this vessel was ligated within the thorax.

Results.—The mucous membranes of both dogs became cyanotic and cold immediately after the ligation. Neither dog roused from the anesthetic, and both expired after five and three hours, respectively. Necropsy revealed an enlarged, turgid liver covered with fibrin and an edematous gall bladder. Clear peritoneal fluid was copious. All the visceral vessels were engorged, with some subserous petechiae in the intestines. The spleen was contracted, the kidneys turgid and the urinary bladder empty.

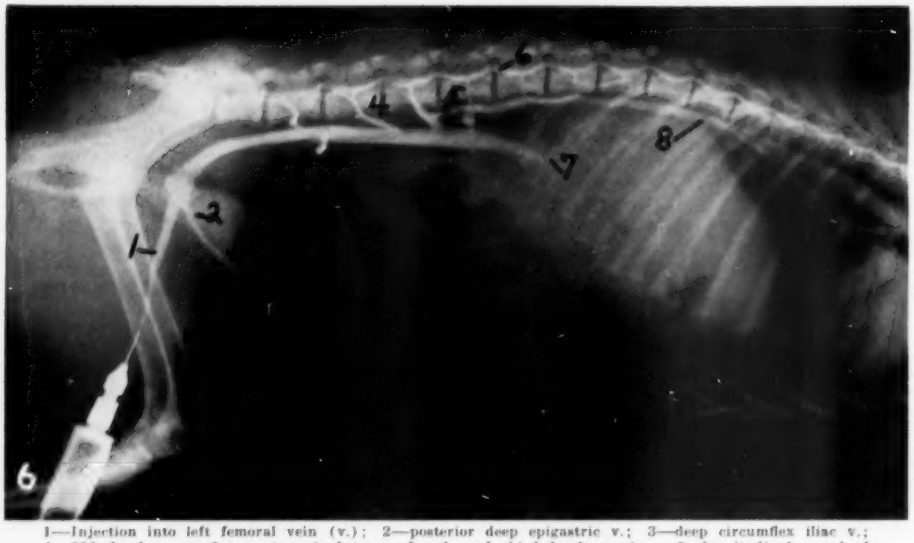
INVESTIGATION 5

Objective.—The purpose was to demonstrate the collateral routes available when the most obvious route of venous return from the head is occluded.



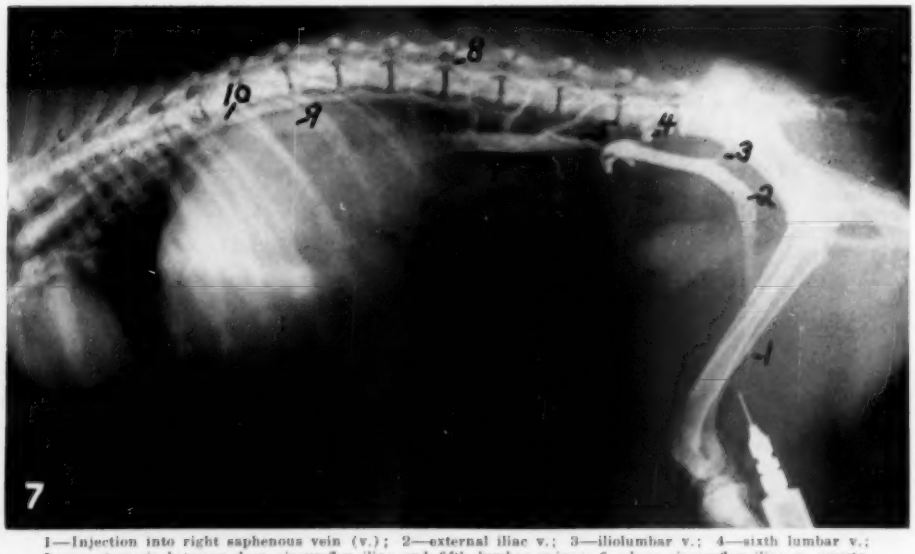
1—Common iliac vein (v.); 2—small channel in caval thrombus which connects with lumbar and deep circumflex iliac veins; 3—point of caval ligation; 4—ureter; 5—fifth lumbar v.; 6—kidney; 7—longitudinal vertebral sinus; 8—azygos v.; 9—point of azygos v. ligation; 10—anterior vena cava; 11—anastomosis between posterior and anterior deep epigastric veins.

Fig. 5—Venograph of dog 6 completed 66 days after ligation of the posterior vena cava and six days after ligation of the azygos vein. Injection was made into the left femoral vein.



1—Injection into left femoral vein (v.); 2—posterior deep epigastric v.; 3—deep circumflex iliac v.; 4—fifth lumbar v.; 5—anastomosis between fourth and third lumbar veins; 6—longitudinal vertebral sinus; 7—point of caval ligation; 8—azygos v.

Fig. 6—Venograph of dog 8 made 18 days after caval ligation anterior to the renal veins.



1—Injection into right saphenous vein (v.); 2—external iliac v.; 3—iliolumbar v.; 4—sixth lumbar v.; 5—anastomosis between deep circumflex iliac and fifth lumbar veins; 6—deep circumflex iliac v. anastomosing with anterior deep epigastric, phrenicoabdominal and intercostal veins; 7—caval segment isolated by ligation; 8—longitudinal vertebral sinus; 9—aorta; 10—azygos v.

Fig. 7—Venograph of dog 8 completed 32 days after caval ligation anterior to and 14 days after caval ligation posterior to the renal veins.

Method.—In dog 11, a 7-year-old male, venographs were made before and immediately after bilateral external jugular ligation. A venograph of dog 12, a 1.5-year-old female, was made before the external jugular veins were ligated 1 inch anterior to the first rib. After 11 days, three venographs were made, all injections of diodrast being made into the angularis oculi vein.

Results.—No edema or other adverse symptoms were observed after the ligation. The normal venous drainage from the head is illustrated (fig. 8). The longitudinal vertebral sinuses were made opaque from the cranial sinuses to the thoracic inlet. After external jugular ligation, the thoracic sinuses were also made opaque. The internal jugular veins became slightly enlarged, but the cervical and thoracic sinuses did not increase in caliber. Postoperatively, the venous return was via the longitudinal vertebral sinuses and the vertebral and internal jugular veins.

INVESTIGATION 6

Objective.—The purpose was to demonstrate the effects upon the subject and to show the collateral routes available when the external iliac veins were occluded.

Method.—Both external iliac veins of dog 13, a 6-month-old female, were ligated just below the common iliac veins. After three weeks, a series of three venographs was made.

Results.—Only a transient skin discoloration of the thighs was noted. Diodrast injected into the femoral vein entered the posterior vena cava via a plexus of vessels which bridged the iliac occlusion (fig. 9). The most direct route was via a large anastomosis between the deep femoral and posterior gluteal veins. The longitudinal vertebral sinuses were not made opaque in this venograph, but when the injection was made into the tail vein, the sinuses were well demonstrated.

INVESTIGATION 7

Objective.—The purpose was to determine the effect upon the dog and to demonstrate the collateral routes remaining when the posterior epigastric veins and the posterior vena cava are both occluded.

Method.—Both external pudendal veins in dog 14, a 1-year-old female, were ligated as they emerged from the inguinal canal. Both posterior deep epigastric veins were ligated 2 inches anterior to the internal inguinal ring. After three weeks, the posterior vena cava was ligated posterior to the renal veins. Eight days later, two venographs were made.

Results.—Only a transient skin discoloration followed the caval ligation. That the longitudinal vertebral sinuses and the much enlarged uterine veins were returning blood from the hindquarters is demonstrated (fig. 10). Dissection revealed that branches of the enlarged posterior superficial epigastric veins were united with the labial and pos-

1—Injection into right angularis oculi vein (v.); 2—cavernous sinus; 3—occipital v.; 4—internal maxillary v.; 5—suboccipital plexus draining into external maxillary v.; 6—anastomosis between 3 and 8; 7—longitudinal vertebral sinus; 8—vertebral v.; 9—internal jugular v.; 10—external jugular veins; 11—costocervical-vertebral trunk; 12—anterior vena cava; 13—posterior extent of diodrast in 7.

Fig. 8—Venograph of dog 12 illustrating the venous return from the head.

terior gluteal veins and drained forward into the internal thoracic veins. This was not evident on the venograph.

INVESTIGATION 8

Objective.—The purpose was to demonstrate the route and speed of venous return from the hindquarters of a normal, anesthetized dog.

Method.—The "ether time" for the tail vein of dog 15, a 6-week-old male, was three seconds. Diodrast was injected, and a radiographic exposure completed within four seconds. Four seconds later a second radiograph was made without further injection.

Results.—The radiographs (fig. 11) illustrate the route taken by the diodrast and show that, within four seconds, the right heart and pulmonary arteries were made opaque. Four seconds later, however, none of the contrast medium was evident in the veins.

INVESTIGATION 9

Objective.—The purpose was to make a venograph while the intra-abdominal pressure could be shown to be elevated by obstruction of the respiratory tract and to demonstrate the route of venous return immediately after caval ligation.

Method.—In dog 16, a 1-year-old male, a catheter was passed into the urinary bladder and connected to a sphygmomanometer of the aneroid type. Respiratory efforts were forced by closing off the nostrils and mouth. The posterior vena cava was then ligated posterior to the renal veins, and a venograph was made 45 minutes later.

Results.—No pressure changes were indicated by the gauge when respirations were forced. The venograph (fig. 12) illustrates the route of venous return immediately after caval ligation.

INVESTIGATION 10

Objective.—The purpose was to compare the routes of venous return from the posterior half of the body under conditions of eupnea and dyspnea.

Method.—Respirations were regulated by a tracheal cannula in dog 17, a 10-year-old male. Venographs were made during eupnea, at the beginning of a deep expiration, and during a severe expiratory effort with the tracheal cannula closed.

Results.—None of the diodrast entered the longitudinal vertebral sinuses during eupnea. From the dorsal vein of the penis, it traversed the internal pudendal veins and entered the heart via the posterior vena cava. There was no difference between the next two venographs and neither differed from the first.

INVESTIGATION 11

Objective.—The purpose was to demonstrate that respiration does influence venous pressure and to produce a venograph during eupnea and dyspnea.

Method.—The left femoral vein of dog 18, a 2.5-year-old male, was cannulated and connected with a mercury manometer by a tube filled with 2 per cent sodium citrate. A tracheal cannula was inserted. Venous pressure readings were made while restricting the passage of air through the tracheal cannula and when digital pressure was applied to the posterior vena cava through the abdominal wall. Two venographs were made, one during eupnea, the other while the dog attempted to exhale when the respiratory tract was obstructed.

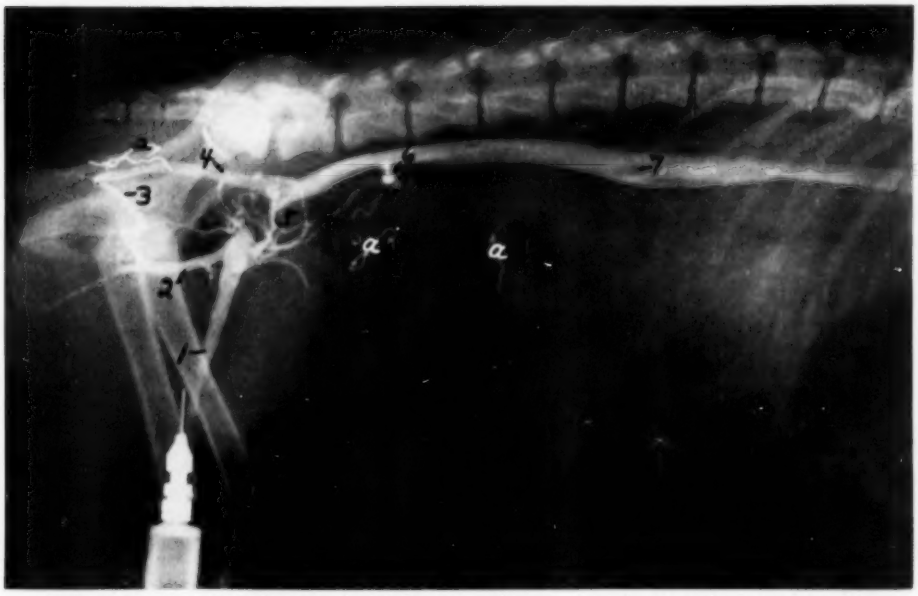
Results.—During dyspnea, the femoral venous pressure rose 20 mm. of mercury. Digital compression of the posterior vena cava raised the pressure 40 mm. The first venograph showed diodrast flowing forward into the posterior vena cava without entering the longitudinal vertebral sinuses. The second duplicated the first, except that this time the fifth and fourth lumbar veins were made opaque. Nothing was seen in the sinuses, however.

INVESTIGATION 12

Objective.—The purpose was to determine the effect of a variety of procedures in raising venous pressure.

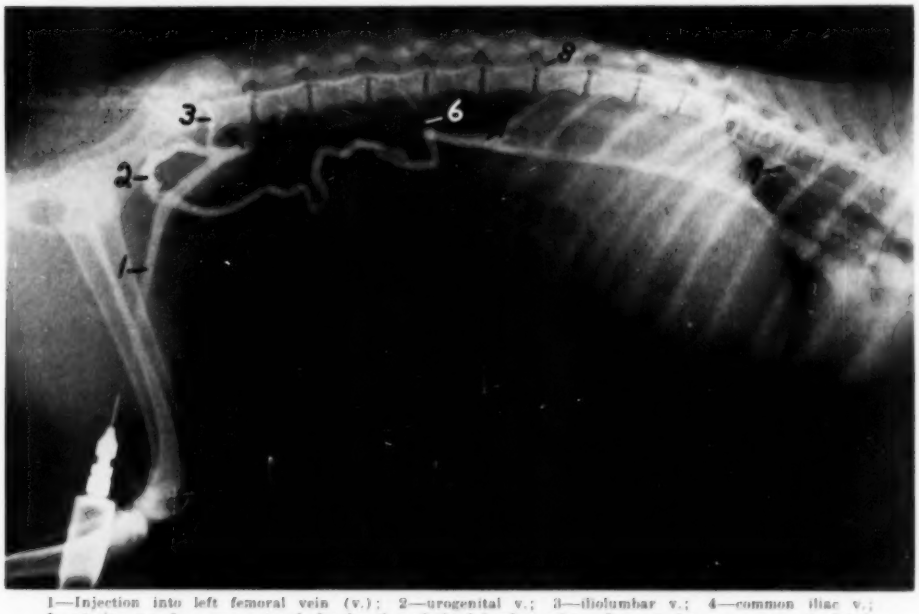
Method.—A tracheal cannula was inserted in dog 19, an 8-month-old female. The left femoral vein was connected to a manometer as in dog 18. First, 5 cc. of adrenaline, 1:10,000, was injected over a four-minute period. Second, expiratory efforts were forced by obstructing the tracheal cannula. Third, digital pressure upon the posterior vena cava was applied through the abdominal wall. Fourth, a compression bandage was tightly placed about the abdomen. Finally, the posterior vena cava was ligated posterior to the renal veins.

Results.—The initial pressure in the femoral vein was 4 mm. of mercury. Adrenaline raised this to 8 mm. During dyspnea, the pressure rose to 16 mm. Digital pressure and abdominal compression with a



1—Left femoral vein (v.)made opaque by injection into saphenous v.; 2—deep femoral v.; 3—anastomosis between posterior gluteal v. and 2; 4—internal iliac v.; 5—plexus of veins bridging ligated external iliac v.; 6—deep circumflex iliac v.; 7—entrance of renal veins into posterior vena cava; a—ingested wire.

Fig. 9—Venograph of dog 13 completed three weeks after bilateral external iliac vein ligation.



1—Injection into left femoral vein (v.); 2—urogenital v.; 3—iliolumbar v.; 4—common iliac v.; 5—uterine v.; 6—common trunk for fourth and third lumbar veins; 7—posterior vena cava; 8—longitudinal vertebral sinus; 9—azygos v.

Fig. 10—Venograph of dog 14 completed three weeks after ligation of the four posterior epigastric veins and eight days after posterior vena cava ligation.

bandage each raised this to 44 mm. Upon caval ligation, the venous pressure rose gradually to 64 mm. of mercury over a three-minute period and remained constant.

INVESTIGATION 13

Objective.—The purpose was to demonstrate radiographically the effect of abdominal compression by a tight bandage upon the venous return via the posterior vena cava.

Method.—A venograph of dog 20, a 2-year-old female, was completed after injecting diodrast into the saphenous vein while a tight bandage compressed the abdomen.

Results.—Neither the longitudinal vertebral sinuses nor their tributaries were made opaque. Only the femoral, external, and common iliac veins and the posterior vena cava were visible.

INVESTIGATION 14

Objective.—The purpose was to make a venographic exposure at the very instant that venous pressure was demonstrated to be raised by abdominal compression.

Method.—The left femoral vein of dog 21, a 2-year-old male, was prepared as in dog 18 for venous pressure readings. The cuff from a blood pressure apparatus used for man was inflated about the dog's abdomen. When the femoral venous pressure reached 56 mm. of mercury, diodrast was injected into the dorsal vein of the penis within the "ether time" interval of five seconds and a venograph was completed. This procedure was repeated for a second venograph, except that the venous pressure was raised to only 50 mm. of mercury, and nine seconds were taken for diodrast injection.

Results.—The first venograph showed complete midlumbar compression of the posterior vena cava. Very little diodrast had found its way into the sinuses. The second exposure showed that some contrast material was passing the area of caval compression and that the anterior gluteal, the iliolumbar, and the sixth lumbar veins were transporting some diodrast into the longi-

1—Injection into right tail vein (v.); 2—internal pudendal v.; 3—internal iliac v.; 4—common iliac v.; 5—confluence of common iliac veins; 6—fifth lumbar v.; 7—longitudinal vertebral sinus; 8—entrance of renal veins into posterior vena cava; 9—right side of heart; a—heart; b—liver; c—right and left kidneys.

Fig. 11—Venograph of dog 15 illustrating route and speed of venous return via the posterior vena cava in a normal animal. Lower half exposed without further injection four seconds after exposure of upper half.

tudinal vertebral sinuses. These were made opaque as far anteriorally as the tenth thoracic segment. The azygos vein was not visible.

INVESTIGATION 15

Objective.—The purpose was to determine the effect of compression and ligation of the posterior vena cava upon cerebrospinal fluid pressure.

Method.—An 18-gauge hypodermic needle was inserted into the subarachnoid space at the foramen magnum of dog 22, a 6-year-old female, and connected to a saline manometer. The posterior vena cava was repeatedly compressed digitally and finally totally ligated.

Results.—None of the above procedures altered the fluid level of the manometer.

DISCUSSION

The posterior vena cava can be ligated below the renal veins without serious effects in man and in many animals.[26, 39, 43] This was substantiated by the results with dogs 1 to 4. Only in dog 1 was there edema present in the hindquarters for more than one day. During periods of postoperative observation up to six months, these dogs, from all outward appearances, were unaffected by the ligations. Skin discoloration was always observed immediately after caval occlusion, but this was no longer evident within a matter of hours.

The part played by the vertebral sinuses in circumventing the ligated posterior vena cava is typified in figures 2, 3, and 4. They were immediately functional routes of venous return (fig. 12). This agrees with findings in human medicine.[1, 2, 38]

Many other veins were routinely seen to enlarge to aid the longitudinal vertebral sinuses in circumventing an occluded posterior vena cava. Among these were the superficial and deep epigastric, the deep circumflex iliac, the phrenicoabdominal, the uterine in the female and, in the male, the ureteral veins. Enlargement of similar vessels in man is reported to occur.[38, 44] Only in dissection of dog 1 was there any indication that the portal system may return some systemic blood in the dog under such conditions. This was never demonstrated radiographically.

The efficiency of all of the collateral routes was influenced in varying degree by the presence of valves. As in man,[20] these

1—Injection into right tail vein (v.); 2—posterior hemorrhoidal v.; 3—internal pudendal v.; 4—middle sacral v.; 5—anterior gluteal v.; 6—common iliac veins; 7—deep circumflex iliac v.; 8—fourth lumbar v. draining into posterior vena cava anterior to ligature; 9—longitudinal vertebral sinus; 10—azygos v.; 11—deep femoral veins; 12—valve in femoral v.

Fig. 12—Venograph of dog 16 completed 45 minutes after caval ligation.

seemed to vary in their degree of competency. Thus, in 1 dog, the superficial epigastric veins may have been enlarged, while in another the deep epigastric veins appeared the more functional. The valves which were present in practically all of the tributaries of the longitudinal vertebral sinuses of the dog[47] did not appear to effectively occlude these vessels when the posterior vena cava was ligated.

Thrombus formation, when it occurred within the ligated vessel, also had a marked effect upon the route and development of collateral channels. Thus, in dog 4, the terminations of both deep circumflex iliac veins were occluded, but extensive anastomoses of their branches with those of the phrenicoabdominal veins indicated the thrombosed posterior vena cava was being bypassed, nevertheless. Caution is required, therefore, in drawing conclusions concerning the routes of venous return which can be demonstrated in a single venograph. Only by means of venographic injections into several different circuits of the venous tree and by careful dissection can conclusions be safely drawn.

The true nature of the numerous, thin-walled veins which appeared as a plexus in the immediate area of the occluded posterior vena cava was not determined. These could well be enlarged vasa venarum described by O'Neill.[36] Clark[6] demonstrated that small vessels already present are capable of enlargement of their lumens, straightening of their courses, and thickening of their walls through the addition of extra-endothelial cells. The various large anastomoses, such as those between the deep circumflex iliac and phrenicoabdominal veins, would appear to have developed in this fashion.

Quiring[38] pictured the azygos vein as another important collateral channel in the event of caval occlusion in man. The part played by it in the dog is also not a small one (fig 6). However, dog 6 was unaffected when both the posterior vena cava and the azygos vein were ligated. By comparing figure 2 with figure 5, one can see that the longitudinal vertebral sinuses were made opaque farther anteriorally after the azygos vein had been ligated. The absence of valves within the sinuses permitted this increased forward flow of blood.

That life itself is endangered when the posterior vena cava is suddenly occluded anterior to the renal veins is well established.[16, 32, 37, 39, 43] This was also stated to hold true for the dog and laboratory animals.[26, 27, 46, 48] Dogs 7 and 8 showed no permanent ill effects from this procedure, however. Despite the extensive thrombus formation which involved even the renal veins in dog 7, blood apparently was adequately returned by the anastomoses between the superficial veins of the kidney[5, 22] and the phrenicoabdominal veins. In dog 8, there was no thrombus formation when the posterior vena cava was ligated both posterior and anterior to the renal veins. Renal blood drained from this segment into the longitudinal vertebral sinuses and the azygos vein (fig. 7). This was an immature dog compared to dog 7. Lew[27] found that rats survived pre-renal ligation in direct relation to their age and maintained that adequate collateral circulation was much more rapidly established in young animals. According to the theory of thrombus formation proposed by Silberberg,[40] this may also explain the difference between the 2 dogs. None of the effects of inadequate renal venous return[3, 41] was observed in either of these animals, however. Both kidneys were of equal size in each and, microscopically, the glomeruli and tubules appeared unaffected. A number of enlarged veins were seen throughout the histological section taken from dog 7.

Ligation of the thoracic posterior vena cava was incompatible with life in dogs 9 and 10. The inadequacy of the longitudinal vertebral sinuses and other collateral channels under these conditions was striking. Others have reported a high mortality associated with this form of ligation in dogs and in laboratory animals.[23, 24, 30] In dog 10, the posterior vena cava had been ligated posterior to the renal veins two weeks before its occlusion anterior to the diaphragm, with the hope that enough collateral development might occur to maintain life. Death resulted, nevertheless.

In dogs 11 and 12, the vertebral sinuses were made opaque farther posteriorally than before the external jugular veins were bilaterally ligated. Batson[1, 2] and others[32, 33] demonstrated upon human cadavers the free communication that exists between the cranial and vertebral sinuses. Gius and Grier[13] described the transient effects of jugular interruption in human patients, but their ligations were done in two stages. The external jugular veins in dog 12 were ligated bilaterally in one operation. Yet, it showed none of the edema or other effects described by Gius and Grier. Although

there was some enlargement of the internal jugular veins, the cervical longitudinal vertebral sinuses and the vertebral veins apparently could carry the increased volume of blood without enlarging their caliber. Neither did dog 22 indicate an increase in cerebrospinal fluid pressure upon caval occlusion, nor did any of the subjects show signs of neurological disturbances.

According to Thebaut and Ward,[43] the collateral circulation in man is much poorer in femoral or external iliac vein ligation than when the posterior vena cava is ligated. Dog 13 showed no ill effect from the ligation of the external iliac vein, and the routes of venous return appeared adequate despite extensive thrombus formation.

Many workers have stressed the importance of the abdominal veins as a collateral channel in caval occlusion.[19, 35, 38] This superficial route also appeared to be of some importance in the dog (fig. 4, 5). However in dog 14, the four posterior epigastric veins were prevented, by ligation, from entering the femoral veins three weeks before the posterior vena cava was occluded. No adverse effects were noted in this dog.

In dog 14 (fig. 10), another important collateral pathway is demonstrated by the large size of the uterine veins. These are also functional in human females.[38] Male dogs were not handicapped, however, because in them the ureteral veins enlarged and united the urogenital veins to the left renal vein and the posterior vena cava.

During the Valsalva experiment extreme increases in intrathoracic and intra-abdominal pressures are produced.[10] Such increased pressures within the body cavities also influence venous pressure and venous return in man and in animals.[9-12, 16-18, 24] According to Batson[1, 2] and others,[13, 32, 33] because of this elevation of venous pressure within the body cavities, blood leaves its usual, more direct route and enters the vast, low pressure system of channels within the spinal canal. Once here, it can flow uninterruptedly on toward the heart or even the cranial sinuses.

The part played by the longitudinal vertebral sinuses as a collateral circuit when the posterior vena cava and other large veins were ligated was seen in dogs 1 to 14. In order to demonstrate the functioning of this system of vessels as a collateral route under more physiological conditions, a number of experiments were performed in an attempt to increase body pressures.

The results in dogs 15, 17, and 18 indicated that the normal course of venous return from the hindquarters of an anesthetized dog is largely by way of the posterior vena cava. This agrees with Batson's[1, 2] observation for the monkey. When he tied a compression bandage about the abdomen of his 2 monkeys, however, venographs indicated that blood was now flowing forward by way of the vertebral circuit as well as the posterior vena cava.

In an attempt to duplicate more physiologically the conditions of the abdominal press than by a compression bandage, respiratory efforts were controlled in 4 dogs. Even though the results in dogs 18 and 19 indicated that this procedure elevated the femoral venous pressure, little diodrast entered the longitudinal vertebral sinuses.

The results in dog 19 indicated that a compression bandage about the abdomen gave the same femoral venous pressure reading as did digital compression of the posterior vena cava through the abdominal wall. When a tight bandage was applied about the abdomen of dog 20, however, there was no great flow of blood into the sinuses. Even when a pneumographic cuff was placed about the abdomen of dog 21 and inflated until the femoral venous pressure was greatly elevated by occlusion of the posterior vena cava, very little diodrast could be seen entering the vertebral sinuses.

It is obvious that great care must be exercised in interpreting such a dynamic process as blood flow from individual venographs. Fluoroscopy and x-ray cinematography would seem to have had definite advantages in certain phases of this work. However, it is felt that the routes taken by blood in its return to the heart were quite accurately determined by multiple points of injection venography, followed by observations made from detailed dissections. Although the anatomy of the longitudinal vertebral sinuses of the dog has been recorded[47] and their place as a collateral route of venous return has been initially explored in this work, their clinical significance as a possible pathway for tumor metastases awaits further study.

SUMMARY AND CONCLUSIONS

1) Twenty-two dogs were subjected to a variety of experimental procedures. Venous trunks were ligated at eight different sites; numerous attempts were made to influence venous return by increasing body pressures. Forty-five venographs of 17 living dogs

were completed, 11 of which are photographically reproduced in this paper. No untoward effects were noticed in any of the dogs in which diodrast® 70 per cent was used.

2) The posterior vena cava, alone, was ligated posterior to the renal veins in 4 dogs. During postoperative observation up to six months in length, no serious effects were noticed.

3) The longitudinal vertebral sinuses appear to be the largest and most immediately available of the collateral routes of venous return after caval occlusion. Under such conditions, the sinuses are capable of enlarging their caliber, as are their tributary and emissary radicles.

4) The number and combinations of other collateral pathways which develop after ligation are markedly influenced by the presence and competency of their valves and by the variable amount of thrombus formation. Most of these collaterals probably result from the enlargement of previously existing, small anastomoses.

5) Ligation of a number of these collateral pathways after previous caval occlusion does not appear to embarrass venous return via the remaining routes.

6) Abrupt occlusion of the posterior vena cava anterior to the renal veins in 2 dogs was not incompatible with survival. In 1 of these dogs, renal venous blood was adequately returned via the vertebral sinuses and the azygos vein even after the posterior vena cava was ligated again, this time posterior to the renal veins.

7) Collateral venous return is inadequate to maintain life when the intrathoracic posterior vena cava of the dog is suddenly occluded. Even when the posterior vena cava in 1 dog had previously been ligated posterior to the renal veins so that collateral vessels had time in which to develop, the animal survived for only three hours after intrathoracic occlusion of the posterior vena cava.

8) Simultaneous ligation of both external jugular veins indicated that the venous return from the head is immediately adequate following such a procedure.

9) The external iliac veins were bilaterally ligated in 1 dog without stasis of blood in the hind limbs.

10) There was no change in the cerebrospinal fluid pressure, in the 1 dog tested, when the posterior vena cava was digitally compressed and finally ligated posterior to the renal veins.

11) Venographs indicate that the normal course of venous return from the hindquarters of an anesthetized dog is by way of the posterior vena cava.

12) Contrary to the results reported in man and in monkey, elevation of the intra-abdominal pressure did not appear to shift an appreciable amount of caval blood flow into the vertebral sinuses of the dogs used in this investigation.

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Macroscopic Anatomy of the Ear of the Dog

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THE ANATOMIST and the clinician have made sporadic studies of the ear of the dog during the past 50 years. However, a review of the literature reveals that a detailed morphological description of the normal ear of the dog is lacking in the standard textbooks. Consequently, it was deemed advisable to reinvestigate and describe in a rather textual manner the macroscopic anatomy of the ear of the dog.

MATERIALS AND METHODS

The gross and subgross structures of the ear were studied from fresh, preserved, and skeletal dog specimens. The age of these ranged from puppies to adults. Whole or part dissections were made on at least 30 heads at Cornell and 20 at Iowa State College to determine the position, form, and relationship of nerves, muscles, and canals of the middle and inner ears. The gross morphology of the membranous labyrinth was studied from a series of sections stained with hemotoxylin and eosin prepared from a fixed (10% formalin), decalcified (5% HCl), and celloidin embedded specimen. Casts of the labyrinth were made by injecting neoprene latex and vinyl resin through the round window of several petrous temporal bones. The oval and round windows were closed with sealing wax in one of which was inserted a 20-gauge hypodermic needle. A 10-cc., rubber-plungered, royal syringe was used to make the injections. Neoprene latex was found to penetrate smaller channels than vinyl resin in these injections. The casts were freed by macerating the bone in 80 per cent HCl. At Iowa, casts of the osseous cochlea and semicircular canals were prepared by placing Wood's metal in the inner ear and centrifuging at high speeds. The temporal bone was later eroded by use of sodium hydroxide leaving a well-formed alloy cast to study and measure. All the illustrations were drawn from specimens prepared specifically for this purpose. Since the dissections were made at different places by different people, they provided an excellent means of checking for accuracy since different approaches were used in their execution. The position of the cochlea was accurately determined from a ventral approach by removing the tympanic bulla and the cap of bone forming the promontory. The spatial relationships of figure 5 were obtained by projecting a drawing of a cast of the osseous labyrinth on a petrous temporal bone in the skull. The particular pyramid (petrous temporal bone) on which the cast was drawn was first removed from the skull; the semicircular canals and aqueducts were flushed with India ink and then sculptured. This bone was then rearticulated and the drawing was made, using like points on the cast and the sculptured pyramid as points of reference.

The small nerves which traverse the middle ear and the one which supplies the m. tensor tympani were studied from fresh specimens by means of a three-powered dissection microscope. (The nerve to the minute m. stapedius, presumably, is from the facial nerve which dwarfs the muscle as it passes in contact with it. In any case, the stapedius nerve was too small to be found by the means used.) The arteries were too small to receive adequate latex injections and were not studied critically.

ANATOMICAL INTERPRETATIONS

The ear, which serves as the organ of hearing, also enables the animal to appreciate the effects of gravity and of movements of the head. It may be divided into three parts: the external ear, the middle ear, and the internal ear.

The external ear consists of the pinna and the ear canal or external acoustic meatus. It is a somewhat funnel-shaped structure which serves to receive air vibrations over a large area and to concentrate them. These vibrations are transmitted to the tympanic membrane (ear drum), which completely closes the deepest part of the external acoustic meatus and thus forms the lateral wall of the middle ear.

The middle ear (fig. 2, 7, 8) consists of the tympanic membrane, the auditory ossicles, the auditory tube, and the tympanic cavity. The tympanic cavity is an excavation in the temporal bone. Rostrally, it communicates with the nasal pharynx by means of the short auditory tube. Atmospheric pressure on each surface of the tympanic membrane is nearly equalized by air in the tympanic cavity which gains ac-

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cess to it by way of this tube, and by air coming directly from the outside through the external acoustic meatus. A jointed bridge comprised of the auditory ossicles and their ligaments serves to transmit air vibrations from the tympanic membrane across the tympanic cavity to the internal ear.

The internal ear (fig. 2, 5, 6) constitutes a closed maze of ducts and sacs, the membranous labyrinth, contained within the osseous labyrinth. The osseous labyrinth while similar in shape is slightly larger than its membranous counterpart. The structures of the internal ear may be separated into three parts: the cochlea, the vestibule, and the semicircular canals. The rostral part is the cochlea, the actual organ where mechanical stimuli are converted to nervous impulses, which, upon reaching the brain, result in audition. The caudal part consists of the three semicircular canals, each in a different plane. Between these is the third part, the osseous vestibule, which contains the utricle and saccule. The semicircular canals and utricle are directly concerned with equilibrium. In 1907, Gray stated that perilymph occupies the narrow space between the endolymph filled membranous labyrinth and the osseous labyrinth.

External Ear.—The external ear (fig. 1) consists of two skin-covered cartilages, the auricular cartilage and the annular cartilage, which form the external acoustic meatus, the passage to the ear drum.

The shape of the pinna, the projecting part of the external ear, varies considerably since most breeds have ears with characteristic shapes. They vary from a small v-shape type carried erect (Scottish and Skye Terriers) to the more lobated semi-

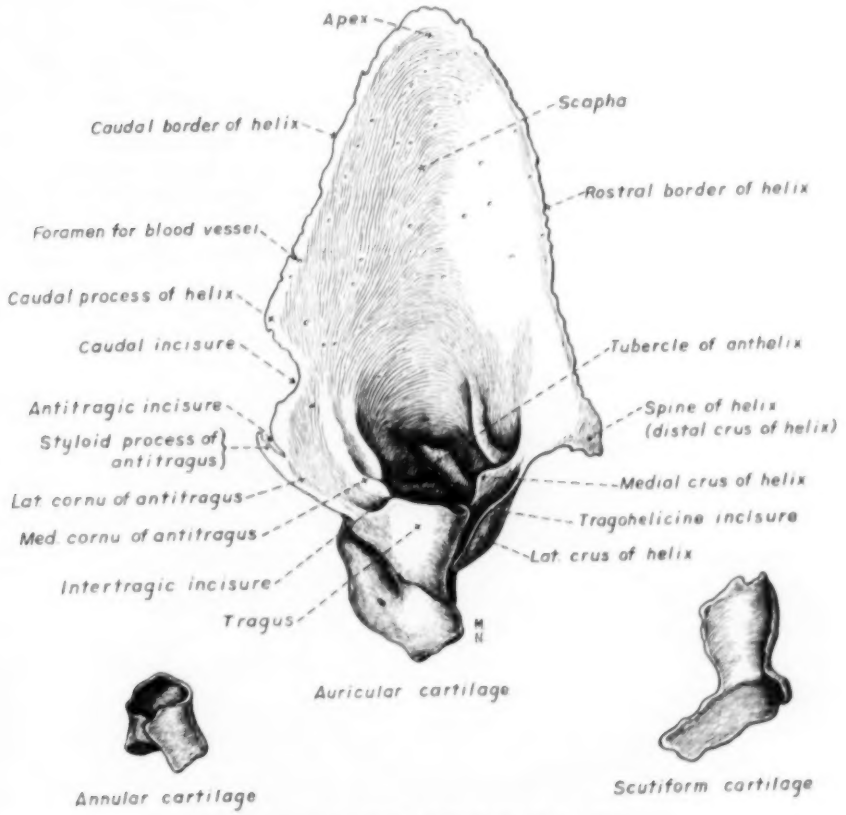

Fig. 1—Cartilages of the right ear of the dog.

erect type (smooth and rough-haired terriers, English Bulldog) to the longer pendant type (spaniels, setters, and hounds).

The ears of domestic animals are more mobile than those of man. So versatile of movement are they, that the rostral edges may become either medial or lateral. Each ear is controlled independently. The ears of members of both Carnivora and Ungulata have been observed facing rostrally on one side and caudally on the opposite side at the same time.

The following general discussion is based upon the pendant type of ear held erect. The isolated cartilages are described separately. The opening of the ear canal faces dorsolaterally. The apex of the pinna points dorsally, the convex or outer surface faces medially, and the concave or inner surface faces laterally. Thus the margins of the pinna are rostral and caudal in the description.

The cartilaginous part of the visible ear or pinna consists of only the auricular cartilage. It is a single sheet of elastic cartilage, thin and pliable at its distal end, thicker and less pliable at its proximal end where it is rolled into a tube. The distal three-fourths of the cartilage is roughly elliptical in outline.

Several anatomical terms stand out in a description of the auricular cartilage. The helix is the free margin of the cartilage.[7] A low transverse ridge, the anthelix, with a prominent tubercle rostrally, is present on the medial wall of the initial proximal part of the ear canal. The concave triangular area between the helix and the anthelix is the scapha.

Opposite the anthelix, forming the lateral boundary of the initial portion of the ear canal, is a relatively dense, irregularly quadrangular plate of cartilage known as the tragus. The tragus curves caudomedially and with the proximal end of the antitragus completes the caudal boundary of the opening into the ear canal. The antitragus is a thin elongated leaf of cartilage located caudal to the tragus and separated from it by an important notch, the intertragic incisure.

The antitragus may be divided into two limbs: the medial cornu and the lateral cornu. They are demarcated caudally by the antitragic incisure. The apex of the lateral cornu ends in a sharp process, the styloid process of the antitragus. Just distal to this, the caudal border presents the cutaneous helicine pouch, which is destroyed when the cartilage is isolated as its medial wall is formed of skin only. A prominent feature of the caudal border of

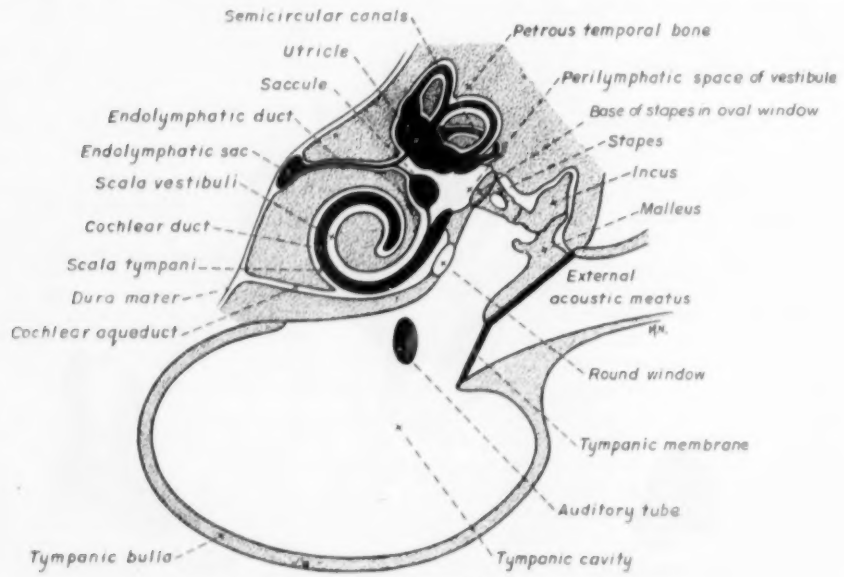

Fig. 2—Diagram of the middle and internal ear of the dog.

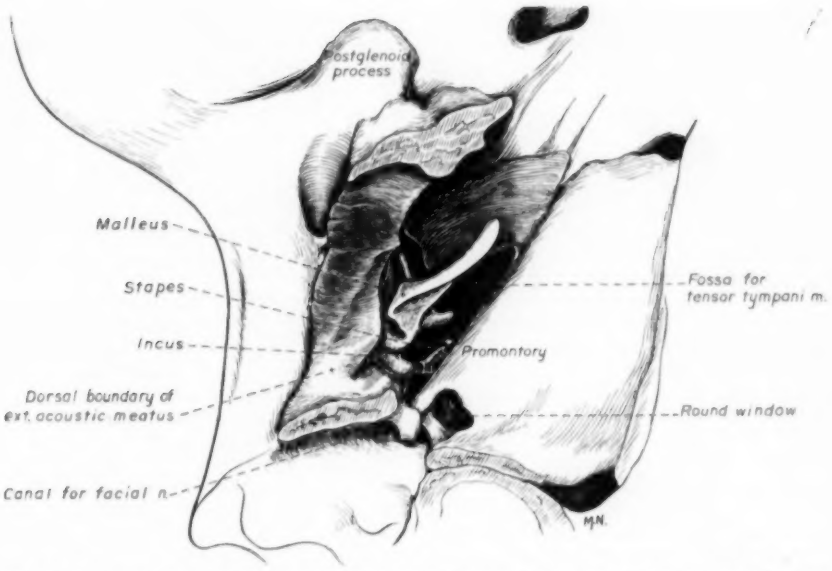

Fig. 3—The ventral aspect of the ossicles of the right ear of the dog. The tympanic bulla has been removed.

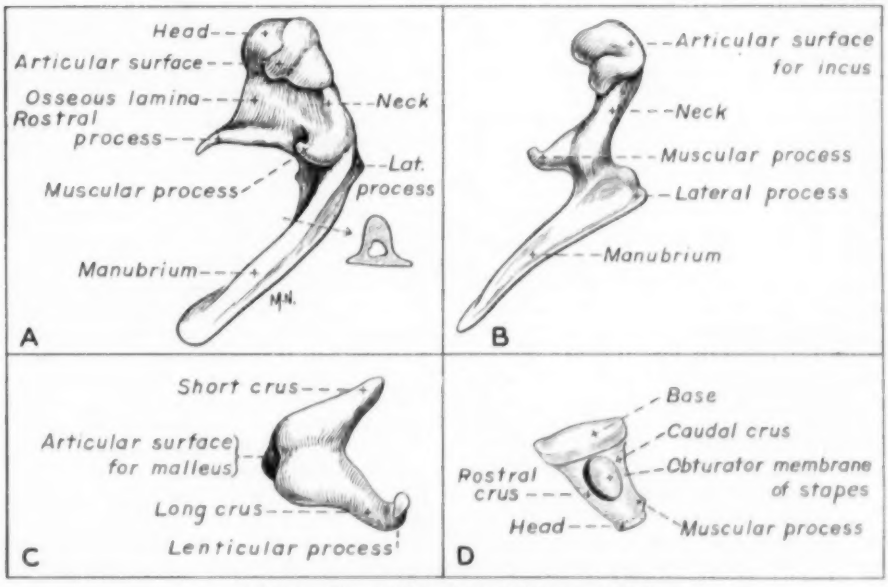

Fig. 4—The ossicles of the right ear of the dog.
A—Malleus, medial aspect and cross section; B—malleus, caudal aspect; C—incus, medial cranial aspect; D—stapes, medial caudal aspect.

the auricular cartilage is the caudal process of the helix located just distal to the cutaneous pouch. Proximal to the caudal process in the region of the cutaneous pouch is the deep caudal incisure or antitragohelicine fissure.

The rostral border of the auricular cartilage is nearly straight. At the junction of the proximal and middle thirds, the spine of the helix or distal crus of the helix is formed by an abrupt cutting-in of this border. Starting just rostral to the tragus and extending rostrally nearly to the spine of the helix is the medial crus of the helix. It is separated from the tragus by the tragohelicine incisure.

Arising rostral to the medial crus of the helix and extending around the medial crus to overlap the rostral border of the tragus is a distinct lamina of cartilage, the lateral crus of the helix.

The concha is the dished area of the auricular cartilage between the scapha and the cartilaginous external acoustic meatus which begins in the auricular cartilage. The diameter of the concha narrows very rapidly toward its proximal part, the lumen of which is continued through the annular cartilage to become continuous with the osseous external acoustic meatus. Numerous foramina occur in the scapha, largely grouped in a semicircle at the junction of the proximal and middle thirds. These serve to transmit vessels from the lateral to the medial side of the cartilage.

A shallow groove is present on the medial surface of the auricular cartilage opposite the anthelix. This is the anthelicine sulcus. The bend, which takes place in the pinna of lop-eared animals, occurs distal to the anthelix, *i.e.*, in the scapha.

Interposed between the auricular cartilage and the external acoustic process is the annular cartilage. It is a narrow sheet of cartilage rolled to form a tube. The free ends meet on the caudal side of the external acoustic process. The diameter of the ear canal is 5 to 10 mm., depending on age, breed, and size of the dog. It is approximately 2 cm. in length. The proximal end of the cartilage overlaps the osseous external acoustic process with which it forms an articulation by means of ligamentous tissue. This process is very short in the dog. The ear canal, therefore, is divided into lateral cartilaginous and medial osseous parts.

The syndesmoses between the incomplete tubes of the auricular and annular cartilages and between the annular cartilage and the external acoustic process provide for great freedom of movement of the pinna. The annular cartilage is smaller than the proximal end of the auricular and is telescoped partly inside of it. The lumen of the portion of ear canal bounded by the annular cartilage becomes somewhat elliptical in outline to conform to the shape of the osseous part of the external acoustic meatus.

The skin lining the scapha and concha shows pigmentation characteristic of the breed. It has, in most individuals, a decreasing amount of hair from the distal to the proximal parts. A few, very fine hairs are found at the entrance of the cartilaginous external acoustic meatus. Many sebaceous and tubular glands are present in the external acoustic meatus. The secretion of both types of glands produces the cerumen or ear wax.[6, 8, 11]

Located in the muscles rostromedial to the ear is a small cartilaginous plate shaped somewhat like a boot. This is the scutiform cartilage. The heel is directed away from the midline, and the toe, rostral to the leg, points medially. It is a sesamoid cartilage intercalated in the preauricular muscles. It forms no part of the external ear. Deep to the scutiform cartilage lies a fatty cushion, the corpus adiposum auriculae. This fatty pillar, which is quite constantly present even in lean dogs, extends over a portion of the superficial surface of the temporal muscle and around the base of the auricular cartilage. It protects the auricular cartilage and somewhat facilitates its movements.[3]

Middle Ear.—Tympanic Membrane.—The tympanic membrane (fig. 7, 8), or ear drum, is a thin semitransparent membrane, ellipitcal in outline, which serves as a wall between the tympanic cavity and the external acoustic meatus. Its longer axis is directed from a rostral to a laterodorsocaudal direction. The shorter diameter is nearly vertical. Its dimensions are subject to marked individual variation averaging 15 mm. by 10 mm. The membrane may be divided into two parts: the pars flaccida and the pars tensa. The pars flaccida is the small triangular portion which lies between the lateral process of the malleus and the margins of the tympanic incisure. The pars tensa constitutes the remainder of the membrane.

The external aspect of the tympanic membrane is somewhat concave due to the traction on the medial surface by the manubrium or handle of the malleus. The most depressed point, which is opposite the distal end of the manubrium, is termed the umbo membranae tympani. When viewed from the external side, a light-colored streak, stria malleolaris, may be seen running dorsocaudally from the umbo toward the pars flaccida. This is caused by the manubrium shining through the tympanic membrane along its attachment. The tympanic membrane of the cat is similar to that of the dog.[10] The manubrium is embedded in the tunica propria and is covered by the epithelium lining the membrane. This membrane is very thin in the center and becomes progressively thicker peripherally (limbus) where it is attached to a fibrocartilaginous circular pad. This in turn is fastened to a definite collar of bone in the external acoustic meatus. This bony collar is incomplete dorsocaudally forming the tympanic incisure.

The tympanic cavity (fig. 2, 7, 8), or tympanum, lies largely within that part of the temporal bone known as the tympanic bulla. It may be divided into: a dorsal portion or epitympanic recess, a middle portion or tympanic cavity proper, and a ventral portion or fundic part within the tympanic bulla.

The epitympanic recess is dorsal to a frontal plane through the osseous external acoustic meatus. It is the smallest of the portions and is occupied almost entirely by the head of the malleus and the incus at their articulation.

The tympanic cavity proper is that portion adjacent to the tympanic membrane. It is irregularly quadrangular in shape, being flattened laterally by the tympanic membrane which forms its wall. Its greatest measurement is less than 1 cm. In the caudal portion, but facing rostrally, is the secondary tympanic membrane closing the round or cochlear window.

The ventral portion, the part within the tympanic bulla, may be compared in shape to the interior of an egg shell having an elliptical opening on the side which faces dorsally. Through this opening, it communicates with the tympanic cavity proper. The long axis of the cavity is about 15 mm. in length and is on an angle of about 45 degrees with the sagittal plane in a caudolateral direction. The width and depth are about equal, measuring 8 to 10 mm.

Within the tympanic cavity is a bony eminence, the promontory, which houses the cochlea; it lies opposite the tympanic membrane medial to the epitympanic recess. The oval or vestibular window which is occupied by the base of the stapes is located on the dorsolateral surface of the promontory just medial to the pars flaccida.

The ostium of the auditory tube is at the rostral extremity of the tympanic cavity proper. The tendon of the tensor tympani muscle descends ventrolaterad through

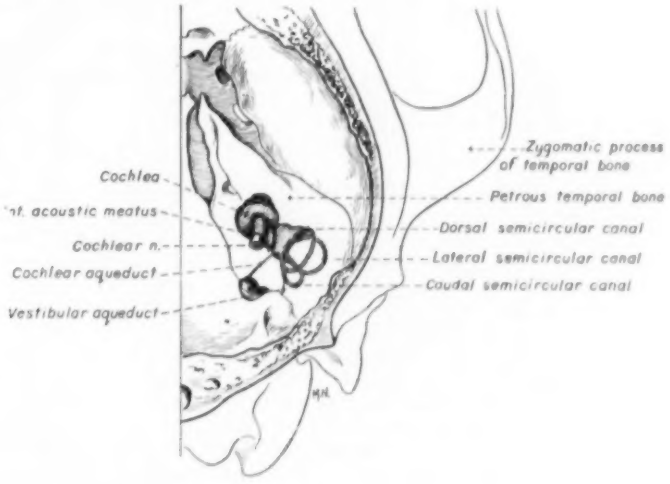

Fig. 5 — Position of the right osseous labyrinth in the skull of the dog, dorsal aspect.

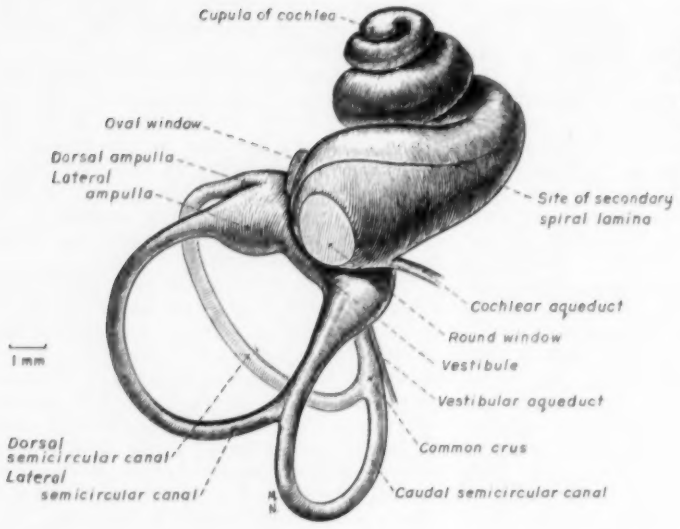

Fig. 6 — The ventral aspect of the latex injection of the right osseous labyrinth of the dog.

a notch in a thin lamina of bone which overlies the muscle. It inserts on the muscular process of the malleus. The ossicles form a short chain across the dorsal part of the tympanic cavity.

The n. chorda tympani (fig. 7, 8), after leaving the facial nerve, passes through the tympanic cavity medial to the malleus to join the lingual nerve. The tympanic plexus arising from the tympanic branch of the glossopharyngeal nerve lies on the promontory and supplies the tympanic mucosa. Other nerves which contribute to this plexus are the small superficial petrosal and the caroticotympanic nerves.

The auditory tube (fig. 2, 8) is a short canal which extends dorsocaudolaterad from the nasal pharynx to the rostral portion of the tympanic cavity proper. Its short bony wall is formed rostrally by the squamous part and, ventrally, its floor is formed by the tympanic part of the temporal bone. The lateral wall which is about 8 mm. long is nearly twice the length of the medial wall. The tube is oval in cross section, with the greater diameter being 1.5 mm. The medial wall of the membranous part of the tube is supported by a plate of hyaline cartilage, the rostral end of which curves medially to form a short hook.

The tensor veli palatini muscle arises in a groove of the petrous temporal bone ventrolateral to the tensor tympani muscle. It supports the lateral wall of the auditory tube. The branch of the fifth cranial nerve which supplies the tensor tympani muscle enters the tympanic cavity in association with the tendon of origin of the tensor veli palatini (fig. 8).

Auditory Ossicles.—The auditory ossicles (fig. 3, 4) are three small bones which form a medium by which air vibrations bombarding the tympanic membrane laterally are transmitted medially across the cavity of the middle ear to the inner ear. The most lateral and largest of the three bones is the malleus. The most medial is the stapes. The handle of the malleus attaches to the tympanic membrane. The base of the stapes is attached to the margin of the vestibular window by a ring of ligamentous fibers. Between the malleus and stapes is the incus which articulates with the other two ossicles.

a) The malleus (fig. 3, 4) is about 1 cm. in length. It consists of a head, a wide thin neck, and a manubrium or handle.

The handle is three-sided in cross section. The side embedded in the substance of the tympanic membrane is wider and smoother than the other two; it is also slightly concave longitudinally. There is a distinct rostral curve in the distal or ventral end of the manubrium so that its termination is in the center of the tympanic membrane. At the base of the manubrium, extending medially and slightly rostrally, is the muscular process of the malleus. This is provided with a tiny hook at its end to which the tensor tympani

muscle attaches. The rostral process or long process is largely embedded in the tympanic membrane. It extends directly rostral from the neck of the malleus arising at the same level as the muscular process. A thin lamina of bone extends from the head, opposite the articulation with the incus, to the rostral process. Opposite the muscular process at an angle of about 90 degrees with the rostral process is the short lateral process. This is the most dorsal attachment of the manubrium to the tympanic membrane. The head of the malleus articulates (diarthrosis incudomalleolaris) with the body of the incus in the epitympanic recess, the most dorsal portion of the tympanic cavity.

b) The incus (fig. 4) is much smaller than the malleus, measuring about 4 mm. long by 3 mm. high. Its shape has often been likened to a human bicuspid tooth with divergent roots. The incus lies caudal to the malleus in the epitympanic recess. The root processes, or crura, are located on each side of a transverse ridge which forms the caudal limit of the recess. The short crus points caudally into the fossa incudis dorsal to this ridge. The long crus is also directed caudally but has a lenticular process which extends rostrally and somewhat medially from its distal end. The head of the stapes articulates with this process.

c) The stapes (fig. 4), the innermost ossicle, is the smallest bone in the body, being only about 2 mm. long. It consists of a head, neck, two legs or crura, a base, and a muscular process. The stapes lies in a horizontal plane with the base facing medially. The base articulates by a syndesmosis with the cartilage which covers the edge of the oval window.

The crura are not solid but are hollowed on their concave or opposed sides. A cross section of a single crus appears as a narrow semicircle of bone. Connecting across from one crus to the other and to the base on each side is a thin connective tissue membrane, the obturator membrane of the stapes. The rostral crus is slightly longer than the caudal crus. Arising from the

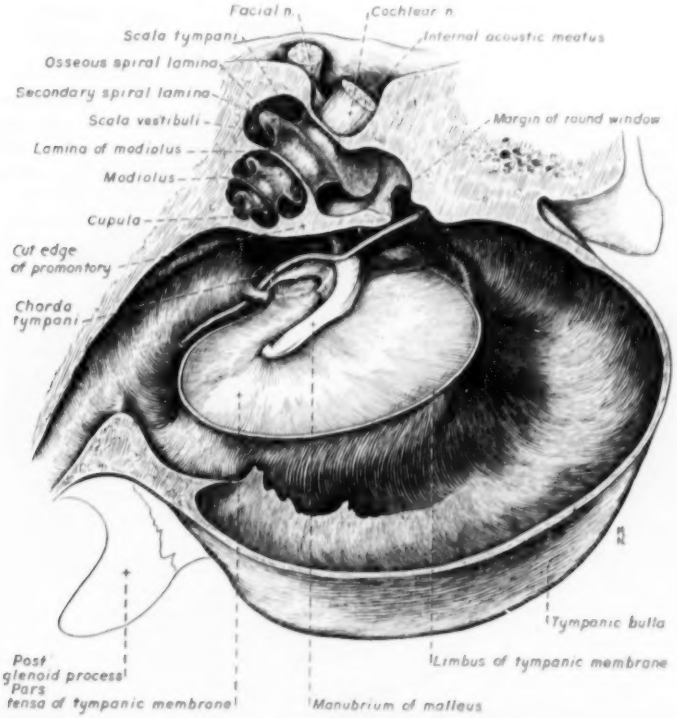

Fig. 7—Medial ventral aspect of the right middle ear of the dog with promontory cut to show cochlea; normal position of the head.

caudal crus near the neck is a minute muscular process which provides attachment for the m. stapedius (fig. 8).

Ligaments of the Ossicles. — Attaching the ossicles to the wall of the tympanic cavity are several ligaments. A short but fairly well-defined lateral ligament of the malleus connects the lateral process of the malleus to the margins of the tympanic notch. The dorsal ligament of the malleus is a somewhat diffuse mass of ligamentous tissue which joins the head of the malleus to a small area on the roof of the epitympanic recess. The rostral ligament of the malleus (fig. 8) is a short ligament attaching the rostral process of the malleus to the osseous tympanic ring just ventral to the canal by which the n. chorda tympani leaves the tympanic cavity.

The body of the incus is attached to the roof of the epitympanic recess by the dorsal ligament of the incus. The caudal ligament of the incus attaches the short crus of the incus to the fossa incudis.

An annular ligament attaches the base of the stapes to the cartilage which lines the oval window. In addition, there are interosseous ligaments which join the ossicles together.

Muscles of the Ossicles.—Two tiny muscles are associated with two of the ossicles. The tensor tympani (fig. 8) is spherical with its rounded base in the fossa tensor tympani. The short tendon of insertion is attached to the hook on the apex of the muscular process of the malleus. Contraction of this muscle tends to draw the handle of the malleus medially, tensing the tympanic membrane. Innervation is by a twig from the mandibular division of the trigeminal nerve.

The stapedius (fig. 8) is the smallest skeletal muscle in the body. Its origin is in an enlargement of the canal for the facial nerve, the fossa musculi stapedis. The body of the muscle lies largely medial to the facial nerve. Its tendon of insertion attaches to the muscular process of the

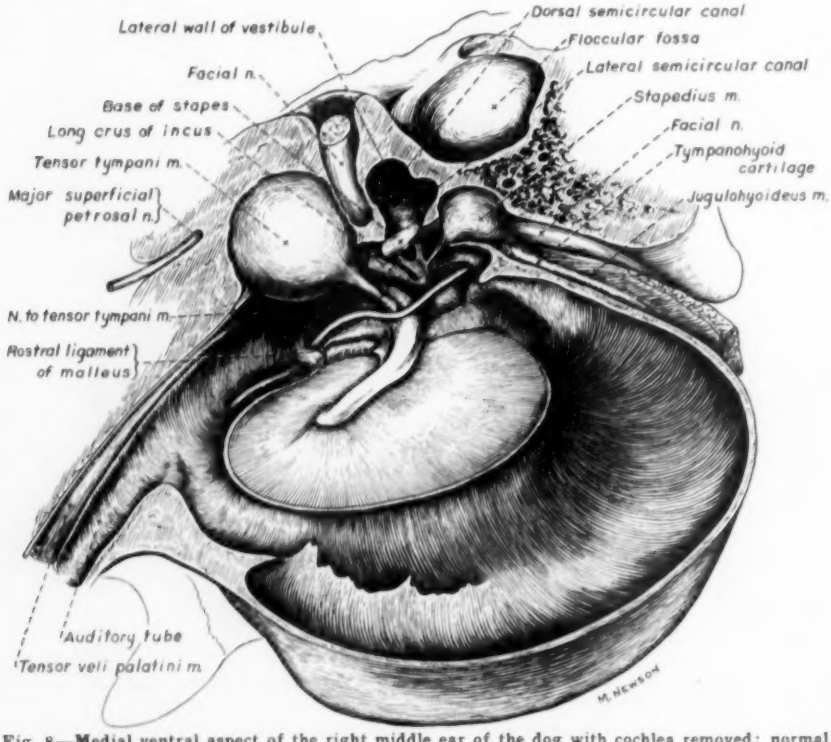

Fig. 8—Medial ventral aspect of the right middle ear of the dog with cochlea removed; normal position of the head.

stapes. Contraction of the stapedius muscle moves the rostral end of the base of the stapes caudolaterally. This muscle is innervated by the stapedial branch of the facial nerve. There is a connection between the stapedius and the insertion of the jugulohyoideus on the tympanohyoid cartilage. This connection may be a remnant resulting from the common origin of the two muscles. According to Wiggers,[12] the tensor tympani and the stapedius, when active, can significantly reduce the efficiency of transmission of tones below 1,000 cycles, and the lower the tone the greater the reduction. Transmission of medium frequency tones (1,300 to 1,800 cycles) is noticeably enhanced while transmission of tones close to 1,100 and those above 2,000 cycles are unaffected by contraction of these intra-aural muscles. According to this author, these observations give more support to the protective theory than to the accommodation theory of the function of the intra-aural muscles.

Internal Ear.—The internal ear (fig. 2, 5, 6) is located within the petrous temporal bone and receives the stimuli which result in hearing and equilibrium. The semicircular canals contain the end organs of the vestibular nerve which conducts impulses resulting in the orientation of the body in space (equilibrium). The cochlea contains the end organs of the cochlear nerve and conducts impulses resulting in hearing. These two parts and the connecting vestibule form the labyrinth. The labyrinth may be divided into an outer osseous labyrinth and the membranous labyrinth which lies within it. Each is a specialized and continuous fluid-containing series of tubes and dilations. Perilymph occupies the bony labyrinth supporting the endolymph-containing membranous labyrinth or cochlear duct, utricle, and saccule, and membranous semicircular canals.

The osseous labyrinth (fig. 5, 6) is about 15 mm. long. It is incompletely divided into three components: cochlea, vestibule, and semicircular canals.

a) The osseous cochlea (fig. 2, 5, 6, 7) is similar in shape to a snail's shell from which it derives its name. It is an osseous tube, or canal, which winds ventrally in a spiral around a hollow bony core, the central axis or modiolus, and ends blindly at the apex or cupula. According to Gray [4] the turns of the cochlea in Carnivora vary from two turns in the seal to about four turns in the weasel. Our observations agree with his that, in the dog, the cochlea makes three and one-fourth turns. The cochlea points ventrorostrally and slightly laterally within the promontory. The osseous spiral lamina (fig. 7) which winds around the modiolus, much like the thread of a screw, nearly bisects the lumen of the spiral cochlear canal into two portions called the scala tympani and the scala vestibuli. The osseous spiral lamina begins within the vestibule and ends at the apex in a free hook-like process, the hamulus. The scala vestibuli communicates with the vestibule and hence is acted on by the base of the stapes in the oval window. The round window is an opening situated near the rostral end of the vestibule by which the scala tympani communicates with the middle ear. In the recent state, the secondary tympanic membrane closes the round window. The membranous cochlear duct completes the separation of the two scalae. The scalae communicate at the apex of the modiolus by a small opening, the helicotrema, formed at the free border of the hamulus. The basal turn of the cochlea is about 4 mm. in diameter and lies close to the medial side of the vestibule. The total height of the cochlea measures about 7 mm. Longitudinal modiolar canals and a spiral modiolar canal serve for the distribution of both blood vessels and nerves to the cochlea. A small canal, the cochlear canaliculus (aqueduct), descends directly ventrad from a point on the ventral wall of the scala tympani near its origin to communicate with the cranial cavity. Perilymph gains access from the subarachnoid space to the vestibule, the cochlea, and the semicircular canals by means of the perilymphatic duct which lies in this small tube.

b) The osseous vestibule (fig. 2, 8) is an irregularly oval space, about 3 mm. in diameter, which communicates with the cochlea rostrally and the semicircular canals caudally. The walls of the vestibule are marked by depressions and ridges which correspond to the various portions of the membranous labyrinth. The medial wall contains two depressions, the caudodorsal one is the elliptical recess which contains the utricle. Rostroventral to it is the spherical recess for the saccule. The vestibular crest separates the two recesses. Several groups of small openings which accommodate the nerves of this region oc-

cur near the recesses. These tiny groups of foramina are called maculae cribrosae.

The lateral wall contains the oval window which is closed by the base of the stapes. Ventral to it is the round window. The vestibular canaliculus (aqueduct), the minute osseous canal for the endolymphatic duct, descends caudoventrally from the vestibule to the caudal surface of the petrous temporal bone. The endolymphatic duct ends in the very small endolymphatic sac just superficial to the dura. The semicircular canals open into the vestibule caudally (fig. 5, 6).

c) The osseous semicircular canals (fig. 5, 6) are three in number, a dorsal, a caudal, and a lateral canal. They lie caudal and slightly dorsal to the vestibule. Each canal describes about two-thirds of a circle in a single plane, each of which is approximately at a 90 degree angle with each of the other two. The segment of a canal proximal to the vestibule is called a crus. Each canal has two crura which communicate with the vestibule (with the exception of the common crus, to be noted later). One crus of each canal has a dilation, the osseous ampulla near the junction with the vestibule. The lumen diameter of the canals averages roughly 0.5 mm. with the ampulla being about twice as large.

In sculptured specimens, the dorsal semicircular canal is seen to surround the flocular fossa, a small but deep depression on the medial side of the petrous temporal bone. This depression is occupied by the flocculus of the cerebellum.[1] The dorsal canal of one ear is roughly parallel to the caudal canal of the opposite ear. The lateral canal of each side occupies a nearly horizontal plane. The dorsal canal is longest. The arc it forms measures about 6.0 mm. across at the widest part. The lateral canal forms an arc which measures about 4.5 mm. while the arc of the caudal semicircular canal is the smallest measuring only 3.5 mm. across in medium-sized dogs. These measurements vary with the size of the dog but, in general, are similar to those found by Honda.[5] The common crus is formed by the nonampullated ends of the caudal and dorsal canals.

The ampullated end of the caudal canal and the nonampullated end of the lateral canal are united for a short distance caudal to the vestibule. The ventral crus of the caudal canal crosses the caudal crus of the lateral canal so that it is ventral to the latter as they enter the vestibule.

The membranous labyrinth is slightly smaller but similar in shape to the osseous labyrinth in which it rests (fig. 2, 6). Surrounding it is the fluid perilymph. Connective tissue trabeculae support the membranous labyrinth and attach it to the osseous wall. Spaces comparable to those in the subarachnoid exist among the trabeculae.

Three regions corresponding to those of the osseous labyrinth are designated. The membranous cochlear duct and membranous semicircular canals simulate their osseous counterparts. The membranous vestibule, however, differs from the osseous vestibule since the membranous part is composed of two saclike structures, the utricle and saccule, which occupy the lumen of the osseous part.

The labyrinthine or internal auditory artery, a branch of the basilar, is the sole supply to the labyrinth.[9] The labyrinthine artery divides into the rostral vestibular artery which supplies the crista of the caudal semicircular canal, and the common crus and a branch which runs in the vestibular aqueduct. A separate branch, which apparently leaves the A. labyrinthi directly, supplies the cochlea. Shambaugh[9] further states that the venous blood from the labyrinth is collected into two trunks, the larger one leaves the internal ear along the cochlear aqueduct and drains the cochlea. The smaller one leaves along the vestibular aqueduct.

The n. acousticus supplies the internal ear. It divides into two branches in the depths of the internal acoustic meatus. These are the cochlear nerve, which supplies the cochlea and the vestibular nerve, which supplies the remaining part of the membranous labyrinth.

SUMMARY

The macroscopic structure of the mammalian ear as contained in the classical descriptions of Bast and Anson [1] and Boas [2] were checked by at least 50 dissections and special preparations of the ear of the dog. Only minor variations were found between their descriptions and our findings. In figure 8, we have shown the relative sizes and forms of the muscles of the auditory ossicles as well as illustrating for the first time, to our knowledge, the nerve supply

to the tensor tympani in the dog. Figures 7 and 8 are unique presentations of the interrelationship of the structures of the middle ear. By the use of modern techniques, the gross anatomy of the ear of the dog has been reinvestigated and illustrated.

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Some Observations on Transplantation of the Canine Kidney

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TRANSPLANTATION of tissue from one animal to another of the same species (homotransplantation) results in the functional survival of the tissue for a limited time only. When a homotransplanted kidney is grafted into the neck, secretion of urine begins promptly, usually continues vigorously for about one day, and then diminishes progressively. In about four days secretion ceases altogether.

The transfusion of blood would seem to be an exception to the rule that homotransplants have such a limited survival period that they are clinically valueless. The survival period of even the animal's own blood is limited.

In acute uremia, kidney transplantation could occasionally be as logical as the transfusion of blood. Therefore, a study of the factors influencing the survival of the homotransplanted kidney was undertaken. In this study, advantage was taken of a new agent—adrenocorticotrophic hormone (ACTH). Since the homotransplanted kidney is invaded by leukocytes, and since ACTH shrinks lymph glands and produces lymphopenia,[1] it was believed that this hormone might aid the survival of the transplanted kidney. Moreover, the transplanted kidney becomes nephrotic, and ACTH is clinically beneficial in nephrosis. Early claims of beneficial effect of ACTH on the homotransplantation of human skin, which suggested that this drug might be beneficial in kidney transplantation, have since been refuted.

REVIEW OF THE LITERATURE

Wu and Mann[2] made a careful study of kidney transplants. The object of their research was to investigate the microscopic changes that take place in transplanted kidneys in the hope of understanding why the homotransplants have but a limited survival period. They found no great changes in autotransplants until the onset of hydronephrosis and infection. Homotransplants showed a picture of nephrosis with normal glomeruli shortly after transplantation.

Williamson[3, 4] reported that the typical histological picture in the homotransplant is "that of acute atypical glomerular nephritis, followed by acute nephritis."

Wu and Mann[2] found that the necrosis of the kidney, which often occurred in the homotransplant, was not necessarily the result of thrombosis at the site of the anastomosis. Instead of being the result of thrombosis, necrosis of the kidney often preceded it and seemed to favor its occurrence.

Simonsen and Sorensen[5] performed homogenous renal transplants on 4 dogs. Two were treated with penicillin and showed no sign of infection on histological examination. The 2 remaining animals were untreated and the transplants became infected. All these animals showed thrombosis of the vessels so that histologically it would be difficult to interpret what was infarction and what was due to the biological reaction to a transplant.

Parkinson and Woodworth[6] have reported on a series of homotransplantations of kidneys in goats. They used the same technique other experimenters have used for dogs with the exception that, instead of vascular suture, they used vitallium tubes to complete the anastomosis. They reported that these homotransplants ceased to secrete urine on about the tenth day. These workers stored kidneys in a deep freeze unit at a temperature of —40 F. for one week before autotransplantation. It was reported that one such kidney was still actively secreting clear urine 30 days postoperatively.

Homotransplantation of the human kidney is being undertaken by several workers. Lawler, West, McNulty, Clancy, and Murphy[7, 8] transplanted a kidney into a woman with renal failure from polycystic disease. Upon exploratory examination of the abdomen following the transplantation, it was found that the kidney had diminished in size, the urethral and pelvic structures were absent and, although the kidney was definitely alive, it was not producing urine. As we have previously mentioned, in our series of experiments kidney homografts functioned for about four days. Reports in the literature prior to the advent of antibiotics occasionally claim a much longer functional period for homotransplanted kidneys, beginning with Carrel in 1905[9]

The Woodruffs[10] have made a notable contribution to the literature of tissue transplantation. However, since they did not use vascular sutures their results may not be quite comparable. They studied the behavior of implanted autografts and homografts of thyroid tissue in two situations, *viz.*, the anterior chamber of the eye and a small subcutaneous pocket prepared beneath the skin of the abdomen.

These workers found that a subcutaneous homograft in guinea pigs is demonstrably antigenic.

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The same is true of a homograft in the anterior chamber, provided that the graft is undergoing destruction. They could not determine whether a surviving homograft in the anterior chamber is antigenic. When the immunizing graft is subcutaneous, the state of immunity is demonstrable in either anterior chamber. When the immunizing graft is in one anterior chamber, the state of immunity is demonstrable in the opposite one. They have some evidence which suggests that the immunizing homograft renders the recipient more resistant to a subsequent graft from the same donor than to a graft from another individual. Immunization of an animal by either a subcutaneous or an anterior chamber graft diminishes the chance of a subsequent homograft "taking." When a homograft of thyroid is established in one anterior chamber for a sufficiently long time, a process of adaptation occurs between graft and host, so that if the graft is made first to the recipient's anterior chamber, and after three months or more is transferred to a subcutaneous site, there is a a good chance of obtaining a successful homograft.

EXPERIMENTAL PROCEDURE

Method.—The method of transplantation employed in these experiments was that described by Markowitz.[11] The renal artery was joined to the common carotid, the renal vein to the external jugular, and the urethral stump was cannulated and led to the outside of the neck. Before performing nephrectomy and after the renal reflection of the peritoneum was stripped from the kidney, the vessels were isolated almost to their source in order to preserve as much of them as possible. Immediately prior to tying off the renal vessels, 0.5 cc. of heparin solution (1:1000) was injected into the renal artery in an attempt to prevent blood clotting within the kidney.

During the time the kidneys to be transplanted (autotransplants and immediate homotransplants) were without a blood supply, they were wrapped in a cotton swab which had been moistened with physiological saline. No other special precautions were taken. After the operation, 100,000 units of crystalline penicillin solution was sprayed over the site of the vascular anastomosis. Daily, for at least one week, 300,000 units of aqueous procaine penicillin was injected intramuscularly.

An ordinary urethral catheter used to insure the flow of urine from the transplanted kidney quickly became plugged with urinary sediment; therefore, polyethylene tubing of a suitable diameter was substituted. It permitted a maximum flow of urine with a minimum tendency to obstruction.

The method of handling refrigerated transplants differed slightly from the foregoing. Instead of being immediately transplanted, the kidneys were placed in a sterile, sealed jar and kept in a refrigerator for 24 hours at 4 C.

Immediate Autotransplants.—Six immediate autotransplants were performed to serve as controls for the immediate homotransplants. Following removal from the abdominal cavity, and before the vascular anastomosis was completed in the neck, the kidneys were without a blood supply for a period which varied from 20 minutes to one hour.

Refrigerated Autotransplants.—Eight refrigerated autotransplants were performed to serve as controls for the refrigerated homotransplants. Following removal from the abdomen, the kidneys to be stored were handled by one of two methods: (1) in sterile physiological saline or (2) in a sterile, dry, sealed jar.

They were then placed in a refrigerator at a temperature of 4 C. for 24 hours before being transplanted.

Immediate Homotransplants.—Three immediate homotransplants were performed.

The Use of ACTH in Homotransplantation.—Two experiments were performed in which the kidney was transplanted immediately after extirpation. In one of these experiments, six days following the successful transplantation, a bilateral nephrectomy was performed on the recipient animal to establish a functional need for the graft (Halstead's principle). The animal was given ACTH at this time and this was continued until the dog was destroyed. The second animal was given ACTH from the time the kidney was transplanted until it was destroyed.

Refrigerated Homotransplants.—If homotransplantation of the kidney is ever to become clinically practicable, it would be desirable that such a transplant survive refrigeration for a brief time. This thought, as well as the possibility that the entity which prevents indefinite survival of a homotransplant might be lost by refrigeration, prompted the investigation concerning the survival of refrigerated transplants.

Four refrigerated homotransplants were performed. The kidneys were stored in a dry, sealed jar in a refrigerator kept at 4 C. for 24 hours before transplantation.

RESULTS

Although the experiments may be considered to have failed in their ultimate objective, they did bring out two facts: (1) The survival time of a homotransplanted kidney is doubled when a dog receives ACTH. (2) A kidney that has been stored for 24 hours in the refrigerator may be transplanted with some success. These two facts are the essential features of this paper.

One of the dogs in which an autotransplant was performed was permitted to live for five months with the transplanted kidney as the sole excretory organ (fig. 1). On histological examination this kidney and the other autotransplants were essentially normal. There was no evidence of hydronephrosis and, although there were small areas of degeneration, no gross le-

Fig. 1—Dog with kidney transplanted into neck. This kidney functioned for several months as the sole excretory organ. The experiment was terminated arbitrarily to determine the histopathology of the kidney.

sions had developed. The use of penicillin prevented gross infection. Some of the autotransplants appeared to have become infected to a minor degree as indicated by an increased number of neutrophils.

Function of the refrigerated autotransplants was poor in comparison with the immediate autotransplants. Urine was excreted slowly in small amounts and was usually blood-tinged. These kidneys functioned for varying periods, some for 14 days. The average time was 7.6 days. In no case was the kidney removed until it had ceased to function. Histopathologically, these kidneys showed almost complete destruction of the parenchyma.

Immediate homotransplants functioned for an average of 4.5 days. No increase in the length of functional time of the homotransplant was evidenced when penicillin was administered. The histological picture of these kidneys was that of nephrosis.

When ACTH was administered to dogs with homotransplanted kidneys, the length of time for which the kidneys functioned was increased to ten days. This was longer than was anticipated and is an indication that ACTH may help the undertaking. The excretion of urine was slow and the fluid tended to be blood-tinged in spite of the physiological need for function of the kidney. The nephrectomized dog vomited almost from the time of the removal of the kidneys from the abdomen, thus becoming quickly dehydrated in spite of copious drinking of water (uremia).

Microscopically, these transplants did not differ materially from other homotransplanted kidneys. The kidney parenchyma had undergone necrosis and lymphocytes and neutrophils were present in equal numbers. Therefore, ACTH had influenced neither the type of cellular infiltration nor the nephrotic state.

Refrigerated homotransplants functioned for a shorter time (an average of 3.5 days) than did the immediate homotransplant. The immediate homotransplant often excretes large quantities of relatively normal urine. The urine from the refrigerated homotransplants was excreted slowly, was always blood-tinged or even definitely bloody, and the quantity was small. Only a few cubic centimeters was excreted in a day. Histological examination of these kidneys showed nephrosis and destruction of the parenchyma.

DISCUSSION

It is not easy to contribute anything new to the knowledge presently available in this field. Those who have deprecated such investigations overlook the homotransplantability of certain malignant tumors. At a certain stage, a potentially malignant cell develops malignant characteristics to the point where it becomes invasive. Further along in its de-differentiation it apparently loses the individuality factor, without which it no longer invites a relentless onslaught by chemical and cellular defenses when transplanted into a new host. The individuality factor [12, 13] is as much an entity as a virus was before the electron microscope disclosed its shape. In several of the foregoing experiments, an attempt was made to alter or eliminate this individuality factor by keeping the kidney

for 24 hours in an ordinary household refrigerator.

Embryological tissues in general lack a strong individuality factor. Such a factor would render more difficult the intrauterine survival of the embryo which may be regarded as a specialized type of total transplant (parabiosis). Once somatic existence occurs, the individuality factor rapidly becomes manifest to be lost again when a tissue is cultured *in vitro*. For example, tissue cultures of parathyroid may be injected, with functional survival, into a patient with parathyroid tetany. The immature cat yields transplants of adrenal cortex which are functional when transplanted into the eyes of adult hosts.[10]

There is a divergency in the claims for the survival of homotransplants. One explanation for this great difference is the fact that the destruction of a homotransplant depends upon its free access to the blood of the host. Thus, tissues which are implanted without vascular anastomosis survive for a much longer period than the kidney or the heart which are immediately introduced by vascular anastomosis to the blood stream of the host. It follows paradoxically that the more expert is the surgery, the shorter is apt to be the period of survival. A better test for the success of a homotransplant is not the duration of its survival, but its functional activity.

Blood transfusions employed 50 years ago, before the knowledge of blood types was general, resulted in dangerous reactions. Homotransplantation of a kidney today appears in the same relatively immature state. Our experiments lead us to suspect that ACTH should improve the duration of a homotransplant's survival and there is no need to hurry the transference of the organ from the donor to the host.

Technically, the procedure is practical and simple. Relatively soon the grafted kidney becomes incapacitated by the humors of the host. We can do little as yet to offset the stubborn individuality of a tissue, an individuality not only of the species but of sufficient intensity in any given animal to doom the transplanted organ to a brief term of functional survival. The surest way to establish whether individuals are really identical twins is to transplant from one to another. The experiment awaits doing for the armadillo which gives birth to identical (uniovular) quadruplets.

Summary

A series of transplantations of the kidney in dogs are described. Immediate autotransplants functioned indefinitely. Refrigerated autotransplants and immediate and refrigerated homotransplants functioned for a limited time only.

Immediate homotransplanted kidneys produced urine for approximately twice as long when the recipient dog was given adrenocorticotrophic hormone.

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Some Aspects of Infectious Canine Hepatitis Virus in Tissue Culture

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THE KNOWLEDGE that infectious canine hepatitis (ICH) is a disease of major significance among the canine population has increased the need for reliable methods of diagnosis which will aid in differentiating this disease from others producing the so-called "distemper complex."

It has been reported that ICH virus multiplies readily in roller tube cultures of canine renal epithelium.[1,2] The growth of the virus is accompanied by marked cytological changes which are inhibited specifically by immune serum. It has been observed also that after serial passage in cultures of this type the virus became markedly modified in its virulence for dogs,[2] producing little or no clinical signs of disease after peripheral inoculation. These observations have had a twofold effect. They have paved the way for the development of simple, sensitive, and specific methods by which ICH can be diagnosed; and they have opened up a wide field for the investigation of the pathogenesis and prophylaxis of ICH.

It is the purpose of this paper to describe the tissue culture methods which have been used for the study and diagnosis of ICH and to report the results of further studies relating to modification of the virus.

MATERIALS AND METHODS

Reagents.—Phosphate Buffered Saline (PBS)[3].—This solution contained 8.0 Gm. NaCl, 0.2 Gm. KCl, 0.1 Gm. $MgCl_2 \cdot 6H_2O$, 1.15 Gm. Na_2HPO_4, 0.2 Gm. KH_2PO_4, 0.1 Gm. $CaCl_2$, 1,000 ml. H_2O. It had a pH of 7.5.

Trypsin Solution.—This is a 0.33 per cent solution of bacto-trypsin 1:250 (Difco) made up in PBS.

Earle's Balanced Salt Solution.[4]—The solution contained 6.8 Gm. NaCl, 0.5 Gm. KCl, 0.2 Gm. $MgSO_4 \cdot 7H_2O$, 1.0 Gm. glucose, 0.125 Gm. $NaH_2PO_4 \cdot H_2O$, 0.2 Gm. $CaCl_2$, 2.2 Gm. $NaHCO_3$, 0.02 Gm. phenol red. For convenience, a 10-times concentrated stock solution may be prepared without the $NaHCO_3$, which is added at time of dilution.

Lactalbumin Enzymatic Hydrolysate.[5]—This solution was prepared as a 5 per cent solution in Earle's without $NaHCO_3$ and autoclaved at 10 lb. of pressure for ten minutes to dissolve and sterilize. It was stored at −20 C. until needed. The unused portion was refrozen as it remained stable indefinitely. On thawing, a precipitate formed which was dissolved by boiling the solution for a few minutes. Since the pH of this solution was quite acid, 0.2 M NaOH was added before use to bring the pH up to 7.4 to 7.6.

Horse Serum.—All of the horse serum used in this work was obtained from 1 horse and was inactivated at 56 C. for 30 minutes before use.

Nutrient Fluid.—This was used in the cultivation of renal epithelium and consisted of 8 parts of Earle's solution, 1 part 5 per cent lactalbumin, and 1 part horse serum. The final pH was approximately 7.6.

All solutions were made with distilled water passed through a resin exchange column so that the mineral content was at least equal to or less than triple-distilled water. They were filtered through porcelain candles for sterilization and, in order to avoid contamination during handling, penicillin and streptomycin were added in concentrations of 200 units and 0.2 mg., respectively, per milliliter of solution.

Preparation of Roller Tube Cultures.—Cultures were prepared in a manner similar to that of Dulbecco and Vogt[3] and Youngner.[6] The cortex from the kidneys of 3- to 6-week-old puppies was removed and minced with scissors into pieces 2 to 3 mm. in diameter. The pieces of tissue were then thoroughly washed with PBS in a 40-ml. centrifuge tube until the supernatant fluid was clear. The PBS was replaced with freshly prepared trypsin solution (previously warmed to and maintained at 37 C.), in the amount of 15 ml. per kidney. The suspension was transferred to a 250-ml. Erlenmeyer flask containing a bar magnet and agitated (just below the foaming point) by means of a magnetic stirrer for ten minutes at room temperature. The tissue fragments were allowed to settle and the cloudy supernatant fluid was removed, saved, and replaced with fresh trypsin solution. The process was repeated in ten-minute cycles. However, the supernatant fluids containing individual cells and small clumps were pooled in 250-ml. centrifuge bottles, and stored at 5 C. The process was continued until the

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pieces of tissue became white and stringy and the supernatant fluids contained mostly connective tissue and few cell clumps. The pooled fluids were centrifuged at 1,000 r.p.m. for five minutes and the supernatant fluid discarded. The sedimented cells were then resuspended in an amount of nutrient fluid equal to the original volume of fluid in the bottle. This suspension was filtered through three to four layers of sterile cheesecloth to remove pieces of connective tissue to which the cells would otherwise adhere causing the formation of large clumps. The filtered suspension was centrifuged at 600 r.p.m. for three minutes and the sedimented cells were resuspended in nutrient fluid in an amount equal to 5 ml. per kidney. This suspension was transferred to a graduated centrifuge tube and centrifuged again at 600 r.p.m. for three minutes. The amount of packed cells was determined and a 0.2 per cent suspension was made in the nutrient fluid. Care was taken to mix the cells and keep them in a homogeneous suspension before removal since they quickly settled out. By means of a 20-ml. syringe fitted with a 3-inch, 18-gauge needle the suspension was transferred in 1-ml. amounts to pyrex test tubes (16 x 150 mm.), which were closed with stoppers of nontoxic white virgin rubber. By this method, 500 to 600 tubes may be prepared from one pair of kidneys. The tubes were incubated at 35 C. in racks set at an angle so that the fluid covered the lower third of the tubes. After four to five days, solid sheets of epithelial cells formed on the walls of the tubes and the nutrient fluid became acid. The fluid was then replaced by 2 ml. of fresh nutrient fluid and the tubes were ready for use. Further incubation was carried out at 35 C. in roller drums rotated at a speed of 12 to 15 revolutions per hour. If tubes were not used at the time of initial change of fluid, subsequent changes were made at least once a week. These tubes remained usable for at least four to six weeks, after which nonspecific degeneration of the epithelial cells generally occurred.

Preparation of Suspended Cell Cultures.—These were prepared from the kidneys, uterus, testes, and spleen of dogs 1 to 12 weeks of age. The organs were minced into pieces 2 to 3 mm. in diameter and washed with PBS as for preparation of roller tube cultures. By means of a capillary pipette, 10 to 15 pieces of tissue were transferred to 25-ml. Erlenmeyer flasks and 3 ml. of nutrient fluid added. The flasks were then stoppered tightly with white rubber stoppers.

It was found that some of the tissues retained their viability when stored at 5 C. for three weeks. However, all cultures prepared in this manner retained their viability for at least three months when incubated at 35 C., provided the nutrient fluids were changed weekly.

Virus Pools.—The virus used throughout these studies was the strain previously described.[2] Stock virus was prepared in large pools, by one of two methods, each producing equally high titers of virus.

One method involved the inoculation of roller tubes with approximately 10^6 tissue culture infectious doses (t.c.i.d.)$_{50}$ of virus. The tubes were incubated until complete degeneration of the epithelial cells occurred, usually three to five days. The fluids from individual tubes were pooled and centrifuged at 2,000 r.p.m. for ten minutes to remove tissue debris. The virus containing supernatant fluid was distributed in glass ampoules in 1-ml. amounts, sealed, and stored at −70 C. The titer of each lot was determined before use by inoculation of 0.2 ml. of decimal dilutions into sets of four roller tubes containing renal epithelium.

The other method involved the use of suspended cell cultures in which virus was produced in 5-liter Povitsky bottles by a method similar to that described by Farrell *et al.*[3] for the cultivation of poliomyelitis viruses. Approximately 5 Gm. of minced kidney cortex was added to each flask containing 500 ml. of synthetic medium 199.[3] The cultures were incubated on a rocking machine for five days at 35 C., after which the fluids were removed and renewed. The flasks were inoculated with 1 ml. of fluid containing approximately 10^6 t.c.i.d.$_{50}$ of virus and incubation was continued on the rocking machine for seven days. The harvested fluids were treated in the same manner as those from roller tube cultures. The titers of individual lots prepared by both methods varied between $10^{6.7}$ to $10^{7.7}$ per milliliter.

Unless otherwise stated, roller tubes were always inoculated with 0.2 ml. of virus and Erlenmeyer flask suspended cell cultures were inoculated with 0.1 ml.

Neutralization Tests.—All dogs were bled by cardiac puncture before inoculation and two and three weeks after inoculation. Decimal dilutions of serums were made in the tissue culture nutrient fluid, each dilution being mixed with an equal quantity of virus so that 0.2 ml. of serum-virus mixture contained 100 to 320 t.c.i.d.$_{50}$ of ICH virus. Incubation was carried out for two hours at 37 C. after which 0.2 ml. of each mixture was inoculated into four roller tubes containing kidney epithelium. Serum was not tested in a dilution of less than 1:10 since it was found that the serums of a large percentage of puppies contained a low level of passively transferred neutralizing antibody against ICH virus, which was insufficient to protect against the disease. All serums from each dog were tested simultaneously. Final readings were made at five to six days when the control titration showed 100 to 320 t.c.i.d.$_{50}$ of virus to be present.

Isolation and Identification of ICH Virus from Body Tissues and Fluids.—Isolation of the virus was attempted from blood and urine during life and from liver after death. These were inoculated as follows: (1) One drop of peripheral blood was inoculated into each culture tube. (2) Urine was obtained by catheterization, the pH adjusted to 7.4 to 7.6 with 0.2 M NaOH, and 0.2 ml. was inoculated into each tube. (3) A small piece of

liver was ground in a TenBroeck grinder and a 1:50 dilution made in PBS. The suspension was centrifuged at 2,000 r.p.m. for ten minutes and three or four tubes were inoculated with 0.2 ml. of the supernatant fluid. If it was not possible to carry out aseptic procedures in obtaining these specimens, penicillin and streptomycin were added in concentrations of 1,000 units and 1 mg., respectively, per milliliter of specimen.

Identification of the agent recovered in tissue culture was carried out by one of two methods. In one method two sets of tubes were inoculated, one containing either the blood, urine, or liver and the other containing one of these specimens plus 0.2 ml. of specific immune serum. The latter prevented the multiplication of ICH virus, hence no cytological changes were observed in those tubes. If the specimen contained ICH virus, cytological changes were observed in the tubes containing no antiserum. The other method involved the use of the standard neutralization test. After virus was isolated from the tissues, it was identified by mixing with undiluted specific immune serum and observing the tubes for cytological changes.

Complement-Fixation Technique.—This was a modified Kolmer test with incubation being carried out overnight at 5 C. The antigen was prepared in the same manner as regular virus pools, except that after the tissue had grown out, the medium was replaced by solution 199. This eliminated anticomplementary reactions on the part of the antigen which was used in a 1:2 dilution.

Dogs.—Only litter mate puppies 2 to 5 months of age were utilized in the animal inoculation work. Individual litters of not less than 5 dogs were kept in outside isolation pens, separated by a distance of at least 20 feet. Prior to use, at least 1 dog, and frequently several, in each litter were tested for susceptibility to ICH infection by intramuscular inoculation of virulent virus. Unless typical signs of disease developed in these dogs, the litter was discarded.

In later experiments, additional checks on susceptibility of litters were made by carrying out neutralization tests on individual serums.

RESULTS

Propagation of ICH Virus.—It was found that all of the isolates of ICH virus tested were readily propagated in cultures of testis, uterus, and canine renal epithelium but only in the latter did it produce specific, typical cytological changes. The earliest manifestation of these changes was a rounding and swelling of the epithelial cells around the periphery of the cellular outgrowth. In the ensuing 24 hours most of the epithelial cells became rounded and highly refractile, the syncytium breaking up and the cells forming in grapelike clusters (fig. 1). The onset of cytological changes was dependent upon the concentration of virus in the inoculum. The results of a typical titration of one of the pools of virus used during these studies are shown (table 1).

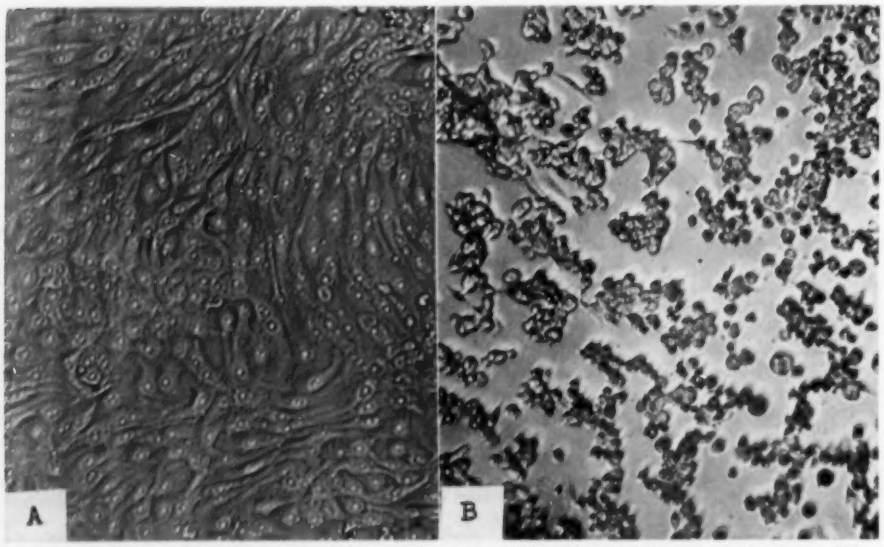

Fig. 1—Roller tube cultures of canine renal cortex; (A) uninoculated control showing solid sheet of epithelial cells and (B) three days after inoculation with 10^4 tissue culture inoculation doses of infectious canine hepatitis virus. x 100.

TABLE 1—Results of Titration of Infectious Canine Hepatitis Virus in Roller Tube Cultures of Dog Kidney

Dilution of virus ($-\log_{10}$)	Cytopathogenic effect (days after inoculation)						
	1	2	3	4	5	6	7
1	0,0,0,0*	3,2,2,2	4,4,4,4	.....	.....	.....	.....
2	0,0,0,0	1,1,1,0	4,4,4,3	4,4,4,4	.....	.....	.....
3	0,0,0,0	0,0,0,0	3,2,2,2	4,4,4,4	.....	.....	.....
4	0,0,0,0	0,0,0,0	2,1,1,1	4,4,3,2	4,4,4,4	.....	.....
5	0,0,0,0	0,0,0,0	0,0,0,0	2,2,1,1	4,4,4,4	.....	.....
6	0,0,0,0	0,0,0,0	0,0,0,0	1,1,0,0	4,4,3,3	4,4,4,4	.....
7	0,0,0,0	0,0,0,0	0,0,0,0	1,0,0,0	2,0,0,0	4,1,0,0	4,4,0,0
8	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0

* Each figure represents one culture tube; 0—no cytological changes; 1—early degeneration involving isolated groups of epithelial cells; 2—definite degeneration, epithelial sheets beginning to break up; 3—widespread degeneration involving majority of cells; 4—complete degeneration.

Attempts to demonstrate cytological changes in cultures of spleen, uterus, and testis inoculated with ICH virus were uniformly negative. Since the cellular outgrowth from these tissues was predominantly fibroblastic, it was concluded that either virus did not propagate in this type of cell or else did so without producing obvious damage.

Although *in vivo* the virus shows a high degree of specificity for the kidney in which it may persist at least six months after infection, the nature of the disease and the finding of inclusion bodies throughout the endothelial system indicate a virus pantropic in nature. It seemed possible, therefore, that the virus was capable of multiplying in tissues other than kidney but without the production of cytological changes. Evidence of multiplication can be obtained either by blind serial passage of the virus or by the demonstration of significant increases of virus in a single passage. The latter method was chosen, and suspended cell cultures of uterus, testis, kidney cortex, and spleen were prepared in triplicate. Each flask was inoculated with 0.1 ml. of fluid containing approximately 1,000 to 10,000 t.c.i.d.$_{50}$ of virus. After one hour of incubation, 0.1-ml. aliquots of fluid were removed and those from replicate sets were pooled. The initial titer of virus in each group was then determined in roller tube cultures of kidney. At seven-day intervals thereafter, the nutrient fluids were removed as completely as possible and the flasks were replenished with 3 ml. of fresh nutrient. The fluids from each group were pooled and virus titers were determined in the same manner as in the original fluids (table 2). It is apparent that multiplication of ICH virus occurred in all but the spleen. The latter was a rather unexpected result in view of the findings with the other tissues, since *in vivo* the spleen is generally rich in ICH inclusions. It is of interest that multiplication of virus occurred in both kidney and testis over approximately the same length of time but continued at least six to eight weeks longer in the uterus cultures. The latter finding may be due to the fact that after three weeks of incubation, the pieces of uterus had become attached to the bottom and sides of the flasks by a monolayer of epithelial-like cells which had begun to grow out of the pieces. During the ensuing weeks the islands of cells coalesced to form solid sheets across the bottom of the flasks.

TABLE 2 — Multiplication of Infectious Canine Hepatitis Virus During a Single Passage in Suspended Cell Cultures of Various Tissues of the Dog

Day after inoculation*	Titer of virus in fluids harvested from cultures of indicated tissues **			
	Uterus	Testis	Kidney	Spleen
0	2.7	3.7	3.0	2.0
7	6.4	5.2	5.4	1.4
14	6.7	5.7	7.0	0
21	6.2	6.5	5.2	.....
28	7.0	6.2	6.2	.....
35	7.2+	5.7	5.4	.....
42	5.7	5.2	6.2	.....
49	6.2	5.0	4.4	.....
56	6.9	4.9	4.2	.....
63	6.4	5.2	4.1	.....
70	5.9	4.7	4.0	.....
77	6.0	3.2	4.0	.....
84	6.2	2.3	4.2	.....
91	6.7	2.4	3.2	.....
98	6.2	1.9	1.4	.....
105	5.7	0	1.3	.....
112	6.0	.....	1.2	.....
119	5.4	.....	1.2	.....
126	5.0	.....	0	.....
133	4.2	.....	.....	.....
140	3.2	.....	.....	.....
147	1.3	.....	.....	.....
154	0	.....	.....	.....

* Fluids were removed totally and renewed at each interval; ** tissue culture infectious dose$_{50}$ per milliliter expressed as the —log of the dilution.

TABLE 3—Results of Testing Tissues of Infected Dogs for the Presence of Infectious Canine Hepatitis Virus

Tissue	Number of specimens from which virus was recovered (days after inoculation)																		Total
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	**	
Liver*	4	5	9	6	5	3	1	1	....	....	....	1	....	....	....	1	....	....	....	4	40
Urine	....	....	....	....	....	2	6	4	8	1	5	1	9	3	2	1	4	2	2	1	51
Blood	1	3	6	3	2	2	1	1	....	....	....	....	....	....	....	....	....	....	....	11	30

* Livers were examined for virus only after the animal had died as a result of infection; ** natural infection, time of infection could not be estimated.

The cells appeared unaffected by the virus and it is probable that this was a factor contributing to continued multiplication of the virus. No cellular outgrowth was observed in flasks containing the other tissues.

Isolation in Tissue Culture of ICH Virus from Infected Dogs.—Although other investigators have reported the recovery of ICH virus from liver,[9] urine and blood [10] of infected dogs, the procedure has involved inoculating these specimens into other dogs with dependence on a clinical response to determine if virus was present. However, if a dog did not respond one could not be certain whether this represented a lack of virus in the original inoculum or immunity of the dog to ICH. The data obtained when tissues from infected dogs were tested in tissue culture for the presence of ICH virus are summarized (table 3).

Where dogs were known to have died from ICH, virus was recovered from the liver in every instance even though no special precautions were observed in taking or preserving these specimens. In several instances, livers were removed after the animals had been dead two days. In general, they were frozen at —20 C. until tested, but several weeks of storage at 5 C. had no effect on the rate of recovery of virus. In the isolation of virus from blood and urine the rate of recovery could not be estimated, since obviously many specimens were tested which contained no virus. However, virus was almost invariably recovered from blood if obtained during the febrile period. Also, where multiple specimens were taken, virus was isolated from the urine of every dog known to have recovered from ICH.

Diagnosis of ICH by Serological Methods.—In the diagnosis of any disease, it is not always possible to obtain specimens for recovery of the etiological agent. For this reason, serological methods have been employed not only to make a retrospect diagnosis but to survey populations for the prevalence of past infection with a particular disease. The complement-fixation test (c.f.) is the only serological method previously used for these purposes in respect to ICH. However, the exacting procedures for preparing antigen and carrying out the test have not always made the use of c.f. practical. Moreover, the neutralization test in tissue culture appeared to be simpler to carry out. The two tests were compared using the serums of 61 dogs (table 4). Among the group of 18 dogs immune to ICH through natural infection, the serums of all had high titers of neutralizing antibody while 4 failed to show c.f. antibody and 2 gave doubtful reactions. All of the group of 43 experimentally infected dogs had neutralizing antibody at least two weeks after infection while only 12 had

TABLE 4—Results of Neutralization and Complement-Fixation Tests for the Demonstration of Antibodies Against Infectious Canine Hepatitis

Group	No. of dogs tested	Complement fixation									Neutralization					
		Preinoculation			2 weeks post.			3 weeks post.			Preinoc.		2 wk. post.		3 wk. post	
		No. neg.	No. doubt.	No. pos.	No. neg.	No. doubt.	No. pos.	No. neg.	No. doubt.	No. pos.	No. neg.	No. pos.	No. neg.	No. pos.	No. neg.	No. pos.
Immune through natural infection	18	4	2	12	....	....	....	....	....	....	0	18	....	....	....	....
Susceptible*	43	43	0	0	16	15	12	5	8	30	43	0	0	43	0	43

* These dogs were inoculated with ICH virus to study antibody formation.

c.f. antibody, 15 gave doubtful reactions, and 16 were negative. By the third week after infection, only 30 reacted positive to the c.f. test, 8 were doubtful, and 5 were still negative.

It is readily apparent that not only was antibody demonstrated earlier by means of the neutralization test, but the sensitivity of this test for the detection of antibody appeared to be superior to the c.f. test.

Modification of ICH Virus.—The virus was maintained by serial passage in roller tube cultures of kidney. The fluids were harvested at two to four-day intervals when at least 50 per cent of the epithelial cells in the cultures showed cytological changes. The undiluted material (containing 10^5 to 10^6 t.c.i.d.$_{50}$ of virus) was then inoculated into a new series of tubes. Virus propagation was maintained in this manner up to the seventieth passage when, in an attempt to establish genetically pure virus, passages were made at limiting dilutions (10^{-5}–10^{-7}). Fluids were harvested up to 12 days after inoculation, but passages were made only with fluids from tubes inoculated with the highest dilutions showing cytological changes.

At various passage levels undiluted virus was inoculated into dogs to test for virulence, and to check on the specificity of the cytological changes observed in tissue cultures (table 5). Between the fortieth and fifty-first passages, the virus apparently became modified to the extent that it no longer seemed capable of producing a lethal infection. By comparison, 25 of 26 litter mates of dogs inoculated with virus from the fifty-first to eighty-seventh passages developed a fatal infection when inoculated with virulent ICH virus that had been maintained by passage in dogs.

Although the modified virus still produced a febrile response in an occasional dog, this too was modified to the extent that it was only of one day's duration in 5 of the 9 dogs which reacted. Further evidence of modification was obtained from results of white blood cell counts (w.b.c.) on 26 of the dogs inoculated with virus from the seventy-fourth passage, including the 6 dogs which showed a febrile reaction. The average low w.b.c. counts of these dogs followed over a period of 14 days postinoculation was 11,800, only 2 dogs having a count of less than 10,000 (7,650 and 8,800). This compared favorably with a group of 10 normal dogs followed over the same period, in which the average low count was 11,900 (1 dog had a count of 7,150). In contrast, 6 dogs inoculated with virulent ICH virus had average counts of 4,400. Since leukopenia is one of the most consistent findings in ICH infection, it would seem that a true modification in virulence had occurred.

When modification of the virus occurred it became apparent that if further work was to be carried out in dogs, it would be necessary not only to prove that the dogs were susceptible to infection prior to inoculation, but had become immune to infection with virulent virus after inoculation with modified virus. Although litter mates of all dogs used in these experiments developed definite clinical signs of ICH after inoculation with virulent virus, it was conceivable that the test animals could have been immune to the disease.

It has been shown that prior to infection with virulent ICH virus the serums of susceptible dogs were devoid of neutralizing antibody and after infection these antibodies were present in high titer. Therefore, neutralization tests were carried out on preinoculation and postinoculation serums of all dogs inoculated with modified virus. Without exception, these dogs showed considerable rises in titer and were immune to challenge with virulent virus. The results of one experiment in which the virus was titered simultaneously in dogs and in tissue culture are summarized (table 6).

TABLE 5—Results of Continuous Passage of Infectious Canine Hepatitis Virus in Tissue Culture Showing Apparent Loss of Virulence for Dogs

Tissue culture passage No.	No. of dogs inoculated*	No. showing clinical signs		No. dead**
		Fever	Corneal opacity	
10	2	2	0	1
11	17	17	2	3
13	5	5	0	3
14	8	8	0	4
23	9	9	3	3
40	2	2	0	0
51	2	0	0	0
54	3	0	0	0
60	4	1	0	0
70	4	0	0	0
74	30	6	1	0
87	12	2	0	0

* Each dog inoculated subcutaneously with 0.2 ml. of undiluted fluid containing 10^5 to 10^6 tissue culture infectious dose$_{50}$.

** Diagnosis of ICH established postmortem either by presence of intranuclear inclusions or recovery of virus from liver, and typical gross pathological lesions of ICH.

The titer in dogs was $10^{-7.2}$ while in tissue culture it was slightly higher, $10^{-8.0}$ per milliliter.

It was obvious that when the modified virus was administered by parenteral routes it was a highly effective immunizing agent. However, it was known from earlier experiments that the modified virus was excreted in the urine, but it was not known whether this virus was modified or virulent and, if modified, whether it would revert to virulence when passed from 1 dog to another by means of natural contact infection. In one series of experiments, 6 dogs were inoculated with virus from the seventy-fourth passage and urine was taken by catheter from each animal beginning three days after inoculation. Between the tenth and fourteenth day after inoculation all dogs excreted virus. Six susceptible, uninoculated dogs placed in contact with those vaccinated at the time of inoculation showed no clinical signs of disease but did develop ICH antibodies five weeks later and were immune to the disease on subsequent challenge. The dogs which received the contact infection were removed to new quarters and placed in contact with another group of susceptible dogs. The virus was maintained in dogs by this type of passage (using 2 to 4 dogs per passage) for five passages. Up to and including the fourth passage, all dogs developed ICH antibodies but no clinical signs of disease. However, 2 of 3 dogs exposed to the fourth back passage dogs developed a severe ICH infection which terminated fatally.

Although the modified virus was innocuous when inoculated parenterally, it obviously represented a potential source of danger to dogs which might come in contact with the urine of vaccinated animals. These results were not totally unexpected since the kidney is the only organ of the dog in which chronic foci of infection are set up. However, kidney was chosen for cultivation of the virus because it was the only tissue tested in which the presence of virus could be detected directly through the cytological changes produced. However, it was felt that if the modified virus were cultivated in other tissues its *in vivo* invasiveness for kidney might be reduced. Therefore, at the eighty-sixth passage level the virus was propagated in suspended cell cultures of canine testis and uterus. Flasks containing either of these tissues were inoculated with about 500 t.c.i.d.$_{50}$ of virus from the previous passage and harvested after seven days' incubation. In this manner, the virus was maintained in testis cultures for four passages and in uterus cultures for ten passages. The titer of virus was comparable to that produced in roller tube cultures of kidney, ranging up to $10^{-7.7}$ per milliliter of fluid. However, in another series of experiments in which 10 dogs were inoculated with virus repre-

TABLE 6—Results of Simultaneous Titration of Modified Infectious Canine Hepatitis Virus in Tissue Culture and in Dogs

Dilution of virus* (−log)	Tissue culture titration	Results with dogs					
		Dog No.	Clinical reaction	Serum-neutralization titer at indicated day after inoculation†			Result of challenge with virulent virus
				0	14	21	
1.7	3,3,3,3**	1	None	<4	1,000	3,200	Immune
		2	None	<4	3,200	4,650	Immune
		3	None	<4	2,150	21,500	Immune
2.7	3,3,3,4	4	None	<4	3,200	3,200	Immune
		5	None	<4	1,000	1,000	Immune
3.7	4,4,4,4	6	None	<4	100	320	Immune
		7	None	<4	32	320	Immune
4.7	5,5,5,5	8	None	<4	3,200	3,200	Immune
		9	F_1	<4	465	2,150	Immune
5.7	5,5,5,6	10	F_1	<4	1,000	2,150	Immune
		11	None	<4	465	2,150	Immune
6.7	6,6,6,6	12	None	<4	3,200	3,200	Immune
		13	None	<4	<4	<4	Died
7.7	7,7,7,N	14	None	<4	<4	<4	320‡
		15	None	<4	<4	<4	3,200‡
8.7	N,N,N,N	16	None	<4	<4	<4	Died

* Based on 1-ml. inoculum for dogs and tissue culture; ** each figure represents day after inoculation that complete cytological changes were noted in one culture tube; † reciprocal of 50% dilution end point tested against 100 to 320 t.c.i.d.$_{50}$ of virus, as calculated by method of Reed and Muench; ‡ typical signs of ICH developed, followed by indicated antibody titer two weeks later; N—no cytologic changes observed; F_1—fever of one day duration.

senting 86 kidney passages plus 10 uterus passages all shed virus, which, however, was modified since nine contacts developed antibodies without showing signs of disease.

Discussion

The need for new diagnostic procedures and methods for the investigation of ICH has long been recognized. The failure of the virus to multiply in embryonating eggs or in animal species other than canine led to the investigation of cultures of homologous tissues. The virus multiplied readily in suspended cell cultures of uterus, testis, and kidney. Sustained propagation in continuous cultures of these tissues could be demonstrated for 98 to 147 days. In roller tube cultures of these tissues, the virus produced no cytological changes either in fibroblast-like cells from kidney, uterus, or testis or in epithelial-like cells from uterus. However, specific cytopathogenic effects were produced in epithelial cells from kidney cortex.

This proved to be a valuable tool, but it was important to determine if the sensitivity of this effect was such that ICH virus could be easily recovered and identified from tissues of infected dogs. The virus was recovered from 30 different specimens of blood, 51 of urine, and 40 of liver. The relatively rapid growth of the virus in tissue culture made identification of the virus possible in as few as two to three days after inoculation, although when minimal quantities of virus were present identification was delayed until the sixth day. Comparative titrations in dogs and tissue culture indicated that both were equally sensitive to the virus. However, one advantage in the use of tissue culture is the fact that the recovery and identification of ICH virus is dependent mainly on whether virus is present, since susceptible cells are exposed directly to the virus. On the other hand, the widespread practice of inoculation of suspected infected material into a new series of dogs involves many unknown factors, including individual immunity of the dogs to ICH and the dependence upon a clinical reaction of the dog (which may or may not be specific) to the inoculated material. Although infected specimens may be examined histologically, this too is a time consuming and somewhat uncertain procedure, since typical inclusion bodies might not be present in the particular sections under examination.

The use of tissue culture makes it possible, with relative ease, to establish a positive diagnosis by isolation of virus from the blood at a time when an animal first shows signs of disease. In those cases in which animals die suddenly without previous illness, the ease and rapidity with which virus is recovered from the liver also makes it possible to establish an early diagnosis so that measures may be taken to prevent the spread of the disease. In addition, the carrier state, which assumes its greatest significance in kennels or other places where large numbers of dogs are kept, can be readily detected by the isolation of virus from the urine.

Perhaps the greatest advantage in the use of tissue culture for the investigation of ICH lies in the fact that any small laboratory can undertake studies on this disease without the use of elaborate isolation procedures and using only the small number of puppies required for preparation of cultures.

For retrospect diagnosis and for the survey of dog populations to determine the prevalence of ICH, the only practical serological test heretofore available has been the c.f. test. This was compared with the tissue culture neutralization test and the latter appeared preferable from the standpoint of sensitivity, ease of performance, and reliability, especially in the early diagnosis of ICH.

Another advantage of the neutralization test over the c.f. test was in the interpretation of negative results. The absence of c.f. antibodies could not be interpreted in terms of susceptibility or resistance to the disease, either because some dogs failed to develop these antibodies or because they appeared quite late after infection. However, it was possible with a high degree of certainty to correlate the results of the neutralization test with previous experience of the animal with ICH infection. All of 143 dogs whose serums contained no neutralizing antibody were subsequently found to be susceptible to ICH, and all showed a rise in antibody two weeks after inoculation. Through the use of this test, it has been possible to screen dogs for use in experimental work by testing their serums prior to inoculation.

Perhaps the most significant finding in

this work was the discovery that the virus had become modified in its virulence for dogs after about 41 serial passages in roller tube cultures of kidney. A febrile reaction was produced in only an occasional dog; 1 of 55 dogs showed a transient corneal opacity while none of 26 dogs tested had a leukopenia. The virus appeared to be an excellent immunizing agent since even minimal quantities produced high levels of neutralizing antibody and solid immunity to reinfection with virulent virus. However, the virus was excreted in the urine of inoculated dogs, although in modified form. Even after 10 passages in suspended cell cultures of uterus, the virus maintained its renal tropism and continued to be excreted in the urine. Although primary contacts of immunized dogs in turn became immune without showing clinical disease, the virus did revert to virulence after four contact passages in dogs.

SUMMARY

1) Techniques are described for the preparation of cultures of canine tissues and the propagation of infectious canine hepatitis (ICH) virus in these tissues.

2) Tissue culture methods for the diagnosis of ICH by virus isolation and serological methods are presented.

3) Further studies on modified ICH virus indicated it to be highly immunogenic after parenteral administration. Modified virus was excreted in the urine, but reversion to virulence took place after four back passages.

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Studies on Experimental Canine Interstitial Nephritis
I. Evaluation of Certain Nephrotoxic Agents

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INTERSTITIAL nephritis is one of the most common conditions seen in dogs, especially those in late adult life. The lesions which characterize this condition have been described by many authors. There appears, however, to be some question as to the various etiological agents possible.[1, 2] Also, very little information can be found as to the functional and morphological development of the lesion. With this in mind, an attempt was made to develop a method of studying the condition experimentally.

MATERIALS AND METHODS

Fourteen dogs were used to test various nephrotoxic agents and to study experimental techniques. This work was conducted in an attempt to find an agent that would give rise in a reproducible manner to kidney lesions resembling those seen in clinical cases of chronic interstitial nephritis, under conditions which would allow repeated functional assessment and morphological study.

All experiments were conducted on dogs less than 3 years of age, weighing 30 to 60 lb. (except 3 puppies, numbers 5, 16, 16A, table 1). Males were used, to facilitate easy catheterization of the urinary bladder, with the exception of 1 dog (7) on which the bladder was exteriorized.

The diet of these animals consisted of dry dog ration except for the two or three days following major surgery, at which time they were fed meat. In addition, each animal was treated with anti-canine distemper and infectious canine hepatitis serums periodically. Each animal was examined periodically for intestinal parasites and treated, if necessary.

Because of the widespread distribution of the disease and the apparent multiplicity of agents capable of producing it,[3-6] it seemed possible that the interstitial lesion might be a common reaction of the canine kidney to a variety of nephrotoxic agents. The following nonliving agents were tried (table 1) in an attempt to produce this condition.

India Ink (Higgins Co.).—This has been used by Altschul[7] to produce nephritis in rats, rabbits, and guinea pigs. Streptomycin sulfate and crystalline penicillin were added to India ink diluted to one half its original concentration with sterile water.

Lithium Carmine (Hartman-Leddon Co.).—This also has been used to produce nephritis by the above author in the same three species. A 5 per cent aqueous solution of the dye was autoclaved just prior to use and administered to 3 dogs.

Desoxycorticosterone Acetate (DCA).—Ingle and Baker[8] and also Addis *et al.*[9] have produced nephritis in rats by administering DCA.

In the present series, 30 mg. per day of DCA in olive oil was administered to an 11-lb. unilaterally nephrectomized puppy. The diet was horse meat, milk, and water. Both fluids contained 1 per cent NaCl. This high protein, high salt diet was used in an attempt to put added stress on the remaining kidney.

A unilaterally nephrectomized litter mate was used as a control animal, which did not receive DCA or added salt.

Nephrotoxic Serum.—Anti-kidney serum has been used to produce nephritis in mice,[10] rats,[11] rabbits,[12, 13] and dogs.[14, 15] The serum used in the present series was prepared by injecting rabbits with saline suspensions of dog kidney. The general technique was that used by Smadel[11] for the production of anti-rat kidney serum. This serum then was injected intravenously into each of 2 unilaterally nephrectomized dogs.

Staphylococcus Toxin.—This toxin has been used to produce nephritis in rabbits,[16-18] cats,[19] and dogs.[20]

The culture of *Staphylococcus aureus* * used was one previously isolated from a Minnesota turkey and believed to produce, predominantly, the necrotoxin. The toxin was recovered from semisolid veal infusion agar plate cultures that had been incubated in an atmosphere containing 20 per cent CO_2 and 80 per cent air. This recovered toxin was roughly titrated as to its effect when injected in 0.2-ml. doses at 30-minute intervals into rabbits. The amount per kilogram of body weight, which would kill the animal in five hours, was arbitrarily designated as 1 rabbit unit. The toxin was injected into 3 animals.

Suspensions of Dead Leptospira Canicola Organisms.—The Leptospira antigen was prepared from a virulent strain of *L. canicola.*** Six-day cul-

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TABLE 1—Experimental Procedures and Results of Urinalysis and Postmortem Examination of Dogs Receiving Various Nephrotoxic Substances

Dog (No.)	Preparation	Substance used	Injection schedule			Urinalysis	Results of postmortem examination of kidneys
			No. of inject.	Route	Dose		
1	Unilateral nephrectomy	India ink	6 at 3-day intervals	i.p.	5.0 ml.	Few hyaline casts and leukocytes seen on second or third day following each injection.	Examined 27 days after first injection; no essential difference between control and exper. kidneys.
11	Unilateral nephrectomy	India ink	1	i.v.	3.0 ml.	None.	Examined 3 hours after first injection; no essential difference between control and exper. kidney.
8	Unilateral nephrectomy	India ink	6 at 3-day intervals	i.p.	5.0 ml.	Few hyaline casts seen on second and third day following each injection.	Examined 30 days after first injection; no essential difference between control and exper. kidney except for few casts in the collecting tubules of latter.
		Lith. carm.	Seventh (day 21)	i.p.	15.0 ml.	Epithelial cells, leukocytes, and casts following injection.	
9	Unilateral nephrectomy	Lith. carm.	6 at 3-day intervals	i.p.	5.0 ml.	Epithelial cells, leukocytes, and casts following injection.	Examined 120 days after first injection; no essential difference between control and exper. kidneys.
7	Bladder explantation	Lith. carm.	First (day 1)	Renal artery	2.0 ml.	Exper. kidney following injection, appearance of albumin and hyaline casts.	Examined 63 days after first injection; exper. kidney enlarged; convoluted tubules separated by spaces containing connective tissue and round cells; large amount perivascular and periglomerular connective tissue; scattered foci of lymphocytes. Control kidney enlarged, otherwise essentially normal.
			Second (day 37)	Renal artery	7.0 ml.	Exper. kidney following injection, increase albumin and hyaline casts, few granular casts. Higher volume and lower sp. gr. than control. Control kidney: albumin.	
			Third (day 61)	Renal artery	15.0 ml.	Exper. kidney following injection, albumin present. Higher volume and lower sp. gr. than control. Control kidney: albumin.	
16	Unilateral nephrectomy	DCA (also fed high-protein, high salt diet)	30	Subcut.	30.0 mg.	Albumin, epithelial cells, leukocytes started appearing day 17, persisted. Hyaline casts and granular casts appeared day 28.	Examined 30 days after first injection; exper. kidney enlarged, capsule peeled with difficulty; foci of peritubular neutrophils along corticomedullary border. Control kidney essentially normal.

16A	Unilateral nephrectomy	None (fed dry kennel ration and milk)	.....	.....	.....	Urine normal throughout experiment.	Examined 30 days after first injection; no essential difference between two kidneys.
4	Unilateral nephrectomy	Nephrotoxic serum	2 intervals of two days	i.v.	20.0 ml.	Few erythrocytes, leukocytes, and hyaline casts seen third day after second injection only.	Examined 6 days after first injection; no essential difference between control and injected kidneys.
5	Unilateral nephrectomy	Nephrotoxic serum	3 intervals of 24 hours	i.v.	10.0 ml.	None made due to difficulty of catheterizing this small male puppy.	Examined 26 days after first injection; exper. kidney, glomerular necrosis and scarring; large numbers of casts in the tubules. Control kidney essentially normal.
13	Unilateral nephrectomy	Staph. toxin	1	Renal artery	0.05 rabbit units	None made; acute experiment.	Examined 23 hours after first injection; exper. kidney swollen with areas of infarction; large amount of necrosis of both tubules and glomeruli; many tubules filled with casts. Control kidney, much congestion and peritubular and periglomerular hemorrhage.
15	London cannula placed on left renal artery	Staph. toxin	2 intervals of 24 hours	Renal artery	0.05 rabbit units	Albumin, epithelial cells, leukocytes and few granular casts following injection.	Examined 48 hours after first injection; exper. kidney swollen and hyperemic, granular on cut surface; diffuse necrosis throughout cortex and much of medulla. Control kidney essentially normal.
18	London cannula placed on left renal artery	Staph. toxin	1	Renal artery	0.002 rabbit units	None made.	Necropsy 48 hours after first injection; exper. kidney swollen and grey color on section; renal artery contained thrombus; necrosis of convoluted tubules but glomeruli intact. Control kidney essentially normal.
20	Unilateral nephrectomy 1 week after a subcutan. dose of antigen	Leptospira antigen	First (day 1)	Subcut.	20.0 ml.	Few epithelial cells and casts of hyaline, cellular and granular composition seen periodically after day 31.	Examined 78 days after first injection; exper. kidney enlarged with few white triangular shaped areas in cortex, where tubules completely replaced connective tissue. Control kidney essentially normal.
			Second (day 31)	Renal artery	20.0 ml.		
			Third (day 41)	Renal artery	12.0 ml.		
			Fourth (day 57)	i.v. Jug. vein	50.0 ml.		
			Fifth (day 72)	i.v. Jug. vein	50.0 ml.		
			Sixth (day 88)	Intracardially	40.0 ml.		

DCA—desoxycorticosterone acetate; i.p.—intraperitoneal; i.v.—intravenously.

tures were examined under a dark-field microscope; those with a count of over 5 organisms per oil-immersion field were treated immediately with formalin to 0.5 per cent final formalin concentration.

Fig. 1—Dog 7 with a surgically explanted urinary bladder. Photograph taken 118 days following explantation and 23 days after the second of three injections of lithium carmine into the left renal artery.

The organisms were left in the solution containing formalin overnight, then washed free of the medium by repeated centrifugation (6,000 r.p.m.) and resuspension. The final suspension of spirochetes represented the recovery of 195 ml. of culture medium contained in a final volume of 20 ml. This antigen then was used to sensitize a unilaterally nephrectomized animal by subcutaneous injection. The sensitizing dose was followed by five other injections via various routes and at various dosage levels.

Surgical Methods.—Two major surgical procedures were associated with this study. One involved the technique of damaging only one kidney and the other was concerned with methods of collecting urine from each kidney separately. In all animals injected with nephrotoxic agents, except dog 7, table 1, pretreatment unilateral nephrectomies were performed, and the excised kidney was studied for later comparison with the retained kidney.

Attempts were made in 4 animals to find a suitable means of injecting directly into the renal artery. Two techniques were tried. The first involved the placement of a permanent London cannula on the renal artery. The second consisted of injecting the agent directly into the artery during laparotomy. In the latter animals, a lateral approach was made between the twelfth and thirteenth rib, and the renal artery isolated and immobilized in an ovariectomy hook. The injection system consisted of a 20-gauge needle bent into a 140-degree angle and connected by polyethylene tubing (PE 200, Clay-Adams Co.) to a three-way stop cock, in turn connected to *(a)* the syringe containing the nephrotoxic agent, and to *(b)* a pressure bottle containing sterile isotonic saline solution. The pressure system prevented the clotting of blood in the needle and made it possible to determine whether the needle was in the arterial lumen before the agent was injected.

The problem of collecting urines separately was solved by explanting the urinary bladder to the ventral body wall. The method used was a compromise between Maluf's[23] technique of explanting only the trigone and Jeanneret's[22] modification of

Fig. 2—Ventral view of surgically explanted bladder of dog 7 (arrow points to left ureteral opening). Photograph taken 118 days following explantation.

explanting the entire bladder wall. The trigone and approximately one third of the bladder wall were retained to be sutured to the wall of the abdomen. One kidney thus was allowed to remain as a control, while the nephrotoxic agent was injected directly into the renal artery of the opposite kidney during repeated laparotomy. The animal operated in this manner (dog 7) remained alert and active throughout the experiments, extending over a period of 16 weeks, and seemed to experience little discomfort (fig. 1 and 2). No signs of ascending infection were observed. This animal was used to test lithium carmine.

Urine specimens were taken for clinical urinalysis from all dogs before treatment and periodically during the experiment.

All dogs, unless otherwise stated, were electrocuted and a systematic necropsy was performed at the conclusion of the experiments. After a rapid examination of the urogenital system, the kidney or kidneys were sectioned *in situ* and the sections were placed in 10 per cent formalin. The kidneys then were removed to allow a closer examination for gross abnormalities. Sections also were taken from liver, spleen, and any organs that appeared abnormal.

Hematoxylin-eosin stains were made of sections from each dog and, in a few cases, sections also were stained with azocarmine.

RESULTS OF PILOT EXPERIMENTS

1) India ink was used in 3 dogs. Although hyaline casts and, in one case, leukocytes were seen in the urine two to three days following each injection given the first 2 animals, no morphological changes were evident in the experimental kidney at necropsy. An attempt was made, using dog 11, to induce an acute reaction by the intravenous injection of the ink solution. Examination of the kidney from this animal showed no morphological changes of an acute nature.

Although vomition and fever followed each injection, indicating a systemic reaction, the only postmorten findings consisted of (1) a widespread discoloration of the peritoneal cavity and reticuloendothelial system with the ink and (2) a thickened intestinal wall containing hypertrophied lymph follicles.

2) Lithium carmine also was tried on 3 animals. Two of these dogs (8 and 9) showed the same general reaction as that produced with India ink. The only notable difference was the absence of a systemic reaction. Necropsy findings again were negative.

The apparently specific effect of carmine on the kidneys of these animals suggested the use of a dog with a surgically explanted urinary bladder. In such an animal (dog 7), three injections were made into the left renal artery using three dosage levels. The first injection resulted in the immediate appearance of albumin in the urine from both kidneys. This persisted throughout the rest of the experiment. Hyaline casts also appeared in the urine from the experimental kidney on the fourth day postinjection and persisted until the second injection.

After the second injection, the albumin increased in the urine from the injected kidney and hyaline casts now appeared in urine from both kidneys, but more in urine from the injected kidney. These casts persisted in diminishing numbers until the third injection, with a few granular casts appearing a few days previous to that injection.

The day before the third injection, the dog was given water, orally, and a timed collection of urine was made from each kidney. At 40 minutes, 176 ml. of urine (sp. gr. 1.000) was collected from the experimental kidney, while the control kidney excreted 143 ml. of urine (sp. gr. 1.004).

The third injection, although twice the previous dose, was followed by no increase in albumin and no casts were passed. A 55-minute collection of urine from the kidneys at this time resulted in the collection of 205 ml. of urine (sp. gr. 1.003) from the experimental kidney and 123 ml. of urine (sp. gr. 1.006) from the control organ. These collections were made by the use of collecting cups placed over the ureteral openings. Just before the animal was destroyed, an additional collection was made; this time no water was given previous to the collection, and the ureters were catheterized up to the pelvis. Twenty-eight milliliters of urine (sp. gr. 1.012) was collected from the experimental kidney during a 40-minute period but only 8 ml. (sp. gr. 1.024) from the control kidney.

At necropsy, the extrarenal changes consisted of intestinal lesions, similar to those seen in animals treated with India ink, and an enlarged spleen with a thickened capsule.

The experimental kidney was larger than the control organ, weighing 80 Gm., versus 68 Gm. for the latter. Adhesions were present in the area of the renal pel-

vis. The artery, vein, and ureter appeared patent and grossly normal.

Microscopic examination of the experimental kidney indicated a generalized interstitial reaction (fig. 3). The cortex had wide interstitial spaces containing scattered foci of lymphocytes. Sections stained for connective tissue showed a fine network around the tubules, and large amounts of periglomerular and perivascular connective tissue. Hyaline casts were present in many convoluted and collecting tubules. Although the epithelium of the convoluted tubules appeared intact, occasional evidence was seen of mitotic activity in the cells, together with cellular debris in the tubular lumens.

The control kidney was essentially normal (fig. 4).

3) Desoxycorticosterone acetate was tried on 1 dog. The animal lost its appetite about the fifteenth day of treatment and continued to lose weight thereafter. Terminally, the dog showed marked muscular tremors, especially when excited. The pupils were dilated and the forelimbs were held in extreme extension. The dog was destroyed at this stage.

Leukocytes were seen in the urine from the seventeenth day until termination and were joined the last two days by hyaline and granular casts.

The control kidney of the litter mate and also the control kidney of the experimental animal, removed before treatment, were both essentially normal, grossly and microscopically.

The capsule of the experimental kidney peeled with difficulty and, on sectioning, a white arciform area could be seen at the corticomedullary border. Microscopic examination showed this to consist of closely placed foci of neutrophils, disposed in peritubular fashion just medial to the corticomedullary border. Neutrophils also were scattered along many of the tubules leading to glomeruli adjacent to the medulla.

4) Two animals were treated with ne-

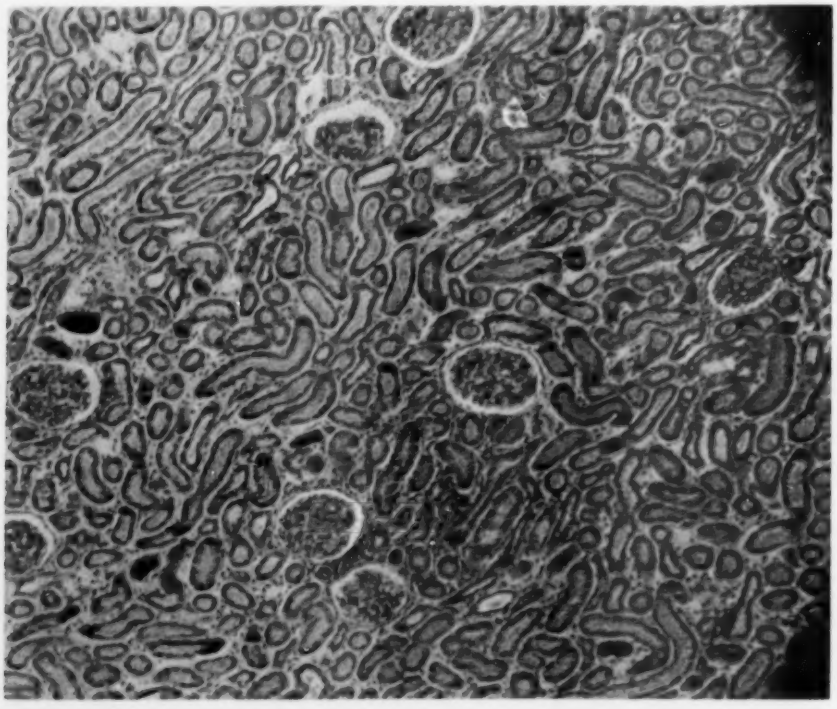

Fig. 3—Experimental kidney of dog 7. Note wide interstitial spaces containing connective tissue and areas of lymphocytic infiltration. Hyaline casts can be seen in some convoluted tubules, but the epithelium is essentially normal. Hematoxylin and eosin. x 100.

phrotoxic serum. Both developed a moist cough and hemorrhagic diarrhea. These symptoms became progressively worse and both animals were destroyed. Very little change was noted in the urine from dog 4; no samples were taken from dog 5. The extrarenal lesions seen in dog 4 consisted of hemorrhagic enteritis and edema of the lung. Dog 5 showed more definite lesions; the gastric mucosa was thrown up into thick edematous folds, 2 to 3 cm. thick in some cases, and the lungs contained small scattered areas of fibrinoid material and lymphocytes.

Dog 4 had no observable renal lesions, grossly or microscopically. The injected kidney of dog 5, however, contained a large number of glomeruli undergoing necrosis and scarring. Hyaline casts were seen in a number of convoluted and collecting tubules. In many areas, the dependent tubules of affected glomeruli were dilated and their epithelia had been replaced with more immature forms.

5) Staphylococcus toxin was injected into the renal artery of 3 dogs. All 3 of these animals showed systemic reactions. Dog 13 was destroyed after 23 hours and dogs 15 and 18 (given lower dosages) at the end of 48 hours. The symptoms and extrarenal changes were those seen in general toxemia.

Albumin, epithelial cells, leukocytes, and a few granular casts were seen in the urine of dog 15 after injection of the toxin. No samples were taken from dogs 13 or 18.

The injected kidneys of these dogs were similar. Some areas of the cortices showed complete necrosis, while other areas showed necrosis only of the convoluted tubules. Many hyaline casts could be seen in the tubular lumens. Dog 18 also had a large thrombus in the renal artery near the site of injection.

All the control kidneys were essentially normal, grossly and microscopically.

6) One animal was given injections (table 1) with killed Leptospira antigen. After the second injection, kidney damage was indicated by the appearance of many hyaline casts and some cellular and granular casts. These were seen following each succeeding injection thereafter.

The dog was kept for 189 days, in an

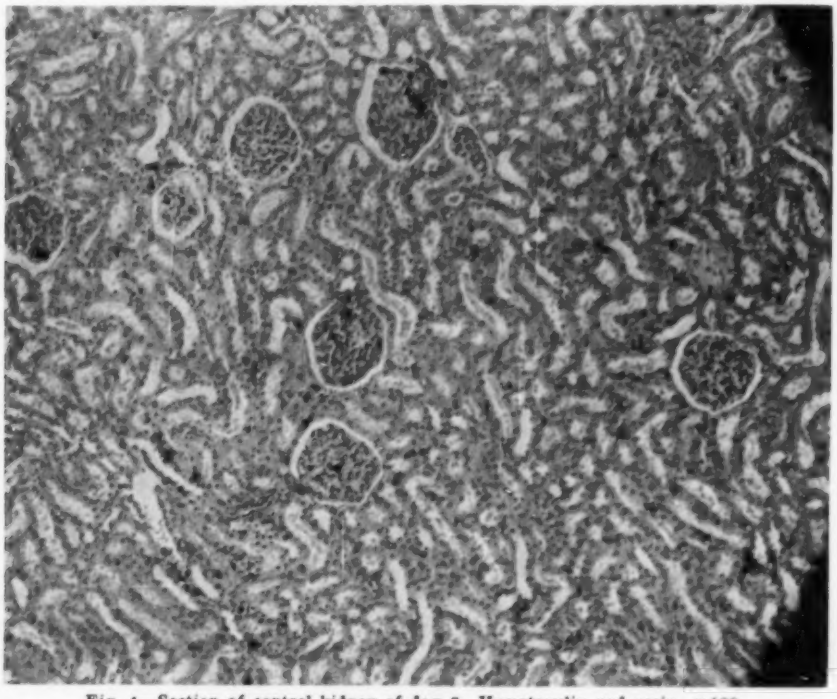

Fig. 4—Section of control kidney of dog 7. Hematoxylin and eosin. x 100.

attempt to allow any lesions to reach a chronic stage. At the time of necropsy, no extrarenal lesions were observed. The injected kidney was grossly different from the control kidney, previously removed. It weighed 84 Gm., compared to 64 Gm. for the control organ, and was pitted on the cortical surface. Sections through these lesions showed triangular white areas with the apexes extending toward the medulla. Microscopically, these resembled infarcts, connective tissue having replaced the tubules, leaving the glomeruli shrunken but intact. The areas adjacent to these lesions contained many swollen glomeruli, in some cases completely filling the Bowman's capsule.

The control kidney was grossly and microscopically normal.

DISCUSSION

A series of experiments was conducted in an attempt to develop procedures and methods for the experimental production and study of interstitial nephritis in dogs. Six nephrotoxic agents were tested for their effectiveness in producing the desired lesion and, of these, lithium carmine and, to a lesser extent, *Staphylococcus aureus* toxin appeared the most promising. The lithium carmine, injected intraperitoneally, seemed to cause a specific reaction in the kidneys of the first 2 animals tested, as evidenced by changes in urinary constituents without evident systemic reactions. Even though no renal lesions were seen, it was felt that this justified a trial at higher dosage levels. For this trial, an animal with an explanted urinary bladder (dog 7) was used. The results indicated a definite effect of the procedure on the injected kidney. This effect appeared to be associated with the injection phase, evidenced by the change in urine composition following each injection. There was no evidence of arterial damage at or near the site of injection or in the kidney itself. Generalized cortical changes seen closely resembled the first stages of an interstitial reaction.

Staphylococcus toxin appeared to be less effective in producing the desired lesion. The widespread cortical necrosis in these dogs and the thrombus in the artery of dog 18 suggested that vascular damage was at least one effect of the toxin. It was quite evident that the toxin should be more dilute. When used at the lower concentrations, the necrosis was confined primarily to the convoluted tubules, leaving the glomeruli and other visible vessels relatively unaffected.

The four other substances tested also did not appear promising as tools for our purpose. The results obtained by using India ink indicated that it did functionally affect the kidney, and also gave severe systemic reactions, although no morphological changes of consequence were seen at necropsy.

Nephrotoxic serum administration was associated with reactions in the kidney, like those seen by others,[14,15] but no interstitial reaction was observed. The systemic reaction, to our knowledge, had not been described previously and possibly was anaphylactic in nature.

The results of the injections of the DCA and Leptospira antigen were of interest. It appeared that these agents might have limited usefulness for more intensive studies.[23] In the present study, the localized nature and shape of the lesions produced by the Leptospira antigen suggested infarction as being their cause.

The results of the trials of various injection techniques and surgical preparations indicated that the general procedure used on dog 7 might be the most effective and useful for further study. This technique, therefore, was the one adopted for use in the later series.[23]

SUMMARY

1) An attempt was made experimentally to produce interstitial nephritis in dogs and to develop a procedure for study of functional-morphological relationships obtaining at several stages of its development.

2) Limited trials were run to test the ability of various nephrotoxic substances to produce interstitial nephritis as follows: India ink, 3 dogs; lithium carmine, 3 dogs; desoxycorticosterone acetate, 1 dog; nephrotoxic serum, 2 dogs; *Staphylococcus aureus* toxin, 3 dogs; *Leptospira canicola* (killed) suspension, 1 dog.

3) Results of these experiments indicated: (*a*) Lithium carmine and, to a lesser extent, *Staphylococcus aureus* toxin showed the greatest promise for the studies desired; (*b*) injection into the renal artery during repeated laparotomy, using an animal with a surgically explanted

urinary bladder, appeared the most promising of the procedures tried; (*c*) high volume, low specific gravity urine, with generalized interstitial renal reaction, was produced unilaterally with lithium carmine.

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Serological Studies of Canine Distemper—Complement Fixation with Spleen Antigens

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PRESENT studies are concerned with the use of complement-fixation tests in the study of canine distemper. Savan and Brandly [1] and Mansi [2] were among the first to use spleen antigens. Mansi, in a more complete report,[3] has demonstrated the value and general specificity of antigens derived from infected spleens when used in complement-fixation (c.f.) tests. How widely such antigens can be employed is unknown. Experience given in the present report is largely restricted to the use of spleens from infected ferrets. Additional data are given for raccoons and skunks. The neutralization test performed in suckling mice, while relatively insensitive, has given added support to serological results obtained.

The canine distemper complex may involve hosts other than domestic animals. Thus, Helmboldt and Jungherr [4] have shown that canine distemper may simulate rabies in wild carnivores and, of particular interest, the work of Adams [5] and more recently of Karzon [6] has shown that canine distemper may bear some relation to the disease in man. In recent communications,[7,8] we have demonstrated that certain strains of distemper virus can lead to jaundice and bilirubinemia. Serological studies described below were largely carried out on experimental animals more fully described in these papers.[7,8]

METHODS

Methods of inoculating animals, preservation of serums, and the isolation of the three strains of distemper virus (DV), the R_1 and R_2 strains from wild raccoons, and the F_1 strain from a spontaneous disease in ferrets, have been given in previous communications.[7,8] Serums used in serological tests were inactivated for a half hour at 56 C.

Complement-Fixation Tests.—Spleens, both normal and infected, were ground with abrasive, diluted 1:10 in c.f. saline, and frozen and thawed four times prior to centrifugation in the cold for one hour at 4,000 r.p.m. The undiluted supernatant from the suspension of infected spleens contained 1 unit of antigen. The technique used for the c.f. test was that described by Bengtson.* Results were checked by Dr. David Lackman of the Serological Unit of the Rocky Mountain Laboratory, using the same technique but with unit volumes of 0.05 ml. instead of 0.2 ml. Titers obtained by this modification were in agreement with those given below. The smaller unit volume was advantageous in conserving antigen.

Neutralization Tests.—A strain of hamster-adapted canine distemper virus, described by Cabasso *et al.*,[10] was obtained * and passed in mice by the intracerebral route. Mouse-brain suspensions which titered 10^{-5} were used for neutralization tests. These tests were performed by intracerebral inoculation of 3-day-old mice with a mixture of serum and virus suspension containing approximately 10 to 20 mouse $l.d._{50}$ doses of DV in volumes of 0.03 ml. Scores were recorded in ten days by counting mice which were dead or moribund. Suckling mice which died within three days of inoculation were not included.

RESULTS

Complement-Fixation Tests of Ferret Serums.—Best results obtained in c.f. tests have been with serums and spleens from adult ferrets infected with the F_1 strain of DV. Of 13 ferrets tested in this series, 11 had c.f. antibodies in postinoculation phase serums, usually taken when animals were moribund. Preinoculation serums, available on 9 of these animals, contained no demonstrable antibodies. Representative findings on 2 ferrets (628 and 1064), bled 11 to 13 days after inoculation, show that both had serum antibody titers of 1:40 (table 1). Ferret 1047 was exceptional in its failure to become ill following inoculation with virulent F_1 virus. Tests showed an antibody titer of 1:10 in preinoculation serum and of 1:160 in serum taken three weeks later. Presumably this animal, immune from an undetermined cause, had experienced a booster effect from the virus inoculated. In contrast to results obtained in ferrets infected with the F_1 strain were those observed in ferrets inoculated with the R_1 strain of DV. Of nine serums tested, only 3 ferrets had c.f. antibodies at the time of death. Ferret 573 (table 1) is an example of this group of three. Preinoculation serums, available from 8 of these ferrets, had no demonstrable c.f. antibodies. In ferret serums (Lederle) used as

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TABLE 1 — Results of Complement-Fixation Tests on Serums of Ferrets Inoculated with Distemper Virus

Infected ferrets			Titers of c.f. antibodies in serum		Strain of DV and tissue	
Designation	Nature of illness	Days after inoculation serum taken	Preinoc.	Postinoc.	Used for original inoculum	Used as source of c.f. antigen
628	Acute	11	0*	40**	F_1 ferret liver	F_1 ferret spleen
1064	Acute	13	0	40	F_1 ferret liver	F_1 ferret spleen
1047	Never ill	21	10	160	F_1 ferret liver	F_1 ferret spleen
573	Acute	13	0	20	R_1 raccoon brain	F_1 ferret spleen
1049	Acute	9	ND	40	F_1 ferret liver	F_1 ferret spleen
			ND	40	F_1 ferret liver	R_1 raccoon spleen
			ND	40	F_1 ferret liver	R_1 skunk spleen
Lederle	.....	Control serums	Normal < 1:5	Immune 1:40	.....	F_1 raccoon spleen

* No fixation at a serum dilution of 1:5; ** figures refer to reciprocal of the highest dilution giving 3+ fixation of complement; ND—not done.

standard, c.f. antibodies were demonstrable in the immune (titer 1:40) but not in the normal serum. As illustrated by ferret 1049 (table 1), c.f. titers obtained with serums of DV-infected animals were of the same order regardless of the source of the spleen (raccoon, skunk, ferret) or the strain of DV involved.

Ferret spleens were tested for presence of c.f. antigens by use of a normal serum and an immune serum obtained from a DV-inoculated ferret. Of 6 ferrets infected with the F_1 strain, spleens from 5 contained c.f. antigen. These results are exemplified by spleens from 4 animals (table 2). Ferret 582, for example, had a titer of 1:20 and ferret 1028 of 1:40 with the known immune serum. Both ferrets also experienced a rise of serum antibodies in the course of their disease. On the other hand, ferret 1049 had a serum antibody titer of 1:40 but no demonstrable spleen antigen. Only one spleen from ferrets inoculated with the R_1 strain of DV was available for testing. This was from ferret 800 (table 2). It gave a titer of 1:40 with the immune serum. Spleens from 2 normal, uninoculated ferrets (1885 and 1897), used as controls, contained no demonstrable antigen. It should be mentioned that ferrets inoculated with two strains of DV of canine origin, as described in a previous report,[5] did not develop demonstrable c.f. antigens in the spleens. Those animals included 2 vaccinated ferrets which succumbed to subsequent challenge with a Lederle strain of DV and 1 ferret (unvaccinated) which became ill following inoculation with the 788 strain of canine origin.[5]

Complement-Fixation Tests of Skunk Serums.—Skunks infected with DV experienced milder illnesses, with longer and more variable incubation periods, than those observed in ferrets and raccoons similarly inoculated. Thus, of 4 skunks inoculated (table 2), 4085 was the only one which, after an illness of three days, became moribund. This animal was likewise the

TABLE 2—Determinations of Presence of Distemper Virus Antigen in Skunk and Ferret Spleens by Means of Complement-Fixation Tests Performed with Normal and DV-Immune Serums

			Titers of c.f. antibodies			
			Test for spleen antigen		Test for serum antibodies	
Source of spleen	Nature of illness	Days after inoculation spleen and serum taken	Normal serum	DV-immune serum	Preinoc. serum	Postinoc. serum
Ferret 582	Acute	10	0*	20**	0	20
Ferret 1028	Acute	13	0	40	0	20
Ferret 800	Acute	12	0	40	0	20
Ferret 1057	Acute	13	0	40	ND	40
Ferret 1049	Acute	9	0	0	ND	40
Ferret 1897	Uninoculated control	....	0	0	....	....
Ferret 1885	Uninoculated control	....	0	0	....	....
Skunk 4162	Inapparent	31	0	40	0	80+
Skunk 4119	Inapparent	19	0	40	0	20
Skunk 4106	Acute	25	0	10	0	20
Skunk 4085	Acute	25	0	10	0	0

* No fixation at a serum dilution of 1:5; ** figures refer to reciprocal of the highest dilution giving 3+ fixation of complement; ND—not done.

TABLE 3—Determinations by Complement-Fixation Tests of Serum Antibodies and Spleen Antigens in Raccoons Inoculated with Distemper Virus

Raccoon No.	Group, based on nature of illness	Days after inoculation spleen and serum taken	Titers of c.f. antibodies: Test for spleen antigens — Normal serum	Test for spleen antigens — DV-immune serum	Test for serum antibodies — Preinoc. serums	Test for serum antibodies — Postinoc. serums
842	Acute	14	0*	20**	0	0
846	Acute	19	0	40	0	0
4098	Acute	8	0	20	0	0
2582	Acute	13	0	40	0	0
R_2	Acute	Natural infection	0	40	....	(0)
1027	Inapparent	24	....	....	0	20
738	Inapparent	42	....	....	0	20
4025	Control	25	0	0	....	....
962	Control	....	0	0	....	....

* No fixation at a serum dilution of 1:5; ** figures refer to reciprocal of the highest dilution giving 3+ fixation of complement.

only one which failed to develop demonstrable antibodies. The remaining 3 skunks had antibody titers of 1:20 to 1:80 in serum specimens taken 19 to 31 days after inoculation. None of the skunks had demonstrable antibodies in preinoculation phase serums. Skunks 4162 and 4119 were considered to have had inapparent infections. Spleens from both of them, when tested for presence of c.f. antigens, gave titers of 1:40 with DV-immune but not with normal serum. Titers of 1:10 were obtained when spleens from the 2 skunks with acute illness (4106 and 4085) were similarly tested.

Complement-Fixation Tests of Raccoon Serums.—No antibodies were demonstrable in preinoculation phase serums from 10 raccoons nor in postinoculation phase serums from 7 of them which became moribund. These animals are illustrated by four examples in the acute group (table 3). A serological diagnosis of distemper in this group became evident when the c.f. test was performed in reverse; that is, when spleens from sick animals were used as antigens against a pair of known normal and DV-immune serums. All of these spleens reacted with the immune serum. Spleens from 2 normal raccoons gave no reactions. The acute group included 1 wild raccoon (R_2) with a spontaneous infection.

Those raccoons which survived infection developed c.f. antibodies. Two of these animals are shown in the inapparent group (table 3).

Control Tests.—Results of control tests performed with 12 human and 11 canine serums, using normal and infected spleen antigens, are shown (table 4). None of the human serums obtained from individuals convalescent from various diseases was positive. Three serums taken from acutely ill dogs had c.f. antibodies against DV. Two of these dogs had clinical distemper, with signs of encephalitis. The third dog had marked jaundice and its serum contained no antibodies against Leptospira. Only 1 of 5 normal dogs which had received avianized vaccine three to five months previ-

TABLE 4—Results of Complement-Fixation Tests for Canine Distemper Performed on Human and Canine Serums Used as Controls

Human case	C.f. antibody titer: With normal ferret spleen	With infected ferret spleen	Serological diagnosis	Dog	C.f. antibody titer: With normal ferret spleen	With infected ferret spleen	Previous history
A	0*	0	Infectious mononucleosis	D_1	0*	10**	Acute clinical distemper
B	0	0	Influenza	D_2	0	20	Acute clinical distemper
C	0	0	Poliomyelitis	D_3	0	60	Acutely ill, jaundiced
D	0	0	Q fever	D_4	0	0	Avianized vaccine 3 to 5 months previously
E	0	0	Q fever (jaundice)	D_5	0	0	
F	0	0	Psittacosis	D_6	0	0	
G	0	0	Rocky Mountain spotted fever	D_7	0	0	
H	0	0	Leptospirosis (Canicola)	D_8	0	20	
I	0	0	Viral hepatitis (clinical Dx)	D_9	0	0	Unvaccinated
J	0	0	Normal control serum	D_{10}	0	5	History unknown
K	0	0	Normal control serum	D_{11}	0	0	History unknown
L	0	0	Normal control serum	....	....	....	.....

* No fixation at a serum dilution of 1:5; ** figures refer to reciprocal of the highest dilution giving 3+ fixation of complement.

TABLE 5—Results of Mouse-Neutralization Tests with Serums of Skunks and Ferrets which Had Experienced a Rise of Complement-Fixing Antibodies after Inoculation with Distemper Virus

Animal	Days after inoculation serum taken	Serum dilution	Results	Animal	Days after inoculation serum taken	Serum dilution	Results
Ferret 5649	Preinoc.	1:2*	11/12**	Skunk 4149	Preinoc.	1:2	10/12
Ferret 5649	17	1:2	1/12	Skunk 4149	19	1:2	2/12
Ferret 5649	17	1:6	0/12	Skunk 4149	19	1:6	0/7
Ferret 5649	17	1:12	0/10	Skunk 4149	19	1:12	0/6
Ferret 5649	17	1:24	1/6	Skunk 4149	19	1:24	0/6
Normal ferrets	(serum pool)	1:2	12/12	Skunk 4162	Preinoc.	1:2	7/13
Ferret 1057	15	1:2	0/12	Skunk 4162	31	1:2	2/12
Ferret 1057	15	1:6	0/12	Skunk 4162	31	1:6	7/7
Ferret 1057	15	1:12	1/12	Skunk 4162	31	....	....

* Final dilution of serum; ** Numerator denotes number of animals dead or moribund from disease; denominator, the number tested.

ously was positive. It was bled five months after vaccination. There was no apparent reason why only 1 of the 5 dogs should have given a reaction.

Neutralization Tests.—A few neutralization tests were performed in suckling mice with the Lederle strain of canine distemper virus. All of these tests were repeated and similar results were obtained. Combined protocols are presented (table 5). It is evident that serums from skunks and ferrets obtained prior to inoculation with one of the bilirubinemia-inducing strains of DV had little or no protective power, whereas serums obtained in an acute phase of illness might protect in a dilution of 1:24. Skunk 4162 had a low titer of neutralizing antibodies in his preinoculation phase serum. As shown in table 2, this animal failed to develop apparent illness.

DISCUSSION

Present studies indicate the value of complement fixation in the diagnosis of canine distemper in carnivores. The test has been made more convenient by the use of small unit volumes. Complement-fixation tests can be used both for detecting antibody by titrating unknown serums against known antigen and for detecting virus by taking the spleen from an acutely ill animal and using it as antigen against known positive serums. Thus, 1 wild raccoon (R_2) with illness resembling rabies infection [5] had no detectable serum antibodies. Distemper antigen, however, was readily demonstrable in his spleen. Histological studies and virus isolations confirmed a diagnosis of distemper.[5] Spleen tissue from 1 dog suffering from clinical distemper gave similar results. With further mouse-adaptation of distemper virus, the neutralization test used in conjunction with complement fixation may furnish a useful tool in working out the natural history of distemper infections in man and animals.

SUMMARY

1) Rises of complement-fixing antibodies have been demonstrated on paired serums obtained from ferrets, raccoons, and skunks inoculated with freshly isolated distemper virus.

2) Antigens prepared from infected spleens, when used in complement-fixation tests, appeared to be specific within the group of carnivores tested.

3) In complement-fixation tests, spleens as well as serum specimens from diseased animals can be used for diagnostic purposes.

4) Neutralization tests performed in suckling mice have proved useful in confirming results obtained by other methods.

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Studies of the Bovine Adrenal Gland. I. The Production of Lipid Accumulation and Other Histological and Cytological Changes in the Zonae Glomerulosae of Calves

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IT HAS BEEN noted repeatedly [1-4] in bovine and ovine ketosis, and also parturient paresis, that marked fatty metamorphosis and other cytological changes occur in the glomerulosa zone of the adrenal cortex. This is of special interest since preponderance of evidence [5-10] indicates that the adrenal zona glomerulosa functions in the regulation of electrolyte balance. These observations were the basis for a series of experiments, two of which are reported here.

MATERIALS AND METHODS

Since the zona glomerulosa is believed to have a function of stimulating the kidney to increase potassium excretion and sodium retention, the following experiments were designed to either hyperactivate or inactivate this zone.

Treatment Schedule, Experiment 1.—For this study, 12 Holstein-Friesian heifer calves, weighing between 35 and 80 kg., were separated into three groups of 4 animals and treated according to the schedule shown (table 1). Except for controls, all animals were treated at four-hour intervals throughout the experiment. The 5 per cent sodium chloride was administered by intrajugular injection at an average rate of 14 ml. per minute. The 2.5 per cent potassium chloride was administered similarly but at an average rate of 10 ml. per minute. Between 250 and 500 ml. of solution was administered at each four-hour interval, depending upon the difficulties encountered. Frequently, potassium chloride injections resulted in bradycardia and oligopnea. In a few instances, it was necessary to use 5 per cent calcium chloride to relieve the symptoms. Usually it sufficed if the injection of potassium chloride was stopped when the symptoms appeared [11] during a given injection period. The desoxycorticosterone acetate (DCA) was administered intramuscularly in 30-mg. doses suspended in 10 ml. of distilled water.

Treatment Schedule, Experiment 2.—In this experiment, 12 Holstein-Friesian heifers, weighing between 115 and 185 kg., were separated into two treatment and two control groups. Animals in the treatment group were drenched four times daily over a period of 72 hours with 24 per cent sodium acetate in tap water, according to the schedule (table 2). Some of the treated and control animals were additionally subjected to water restriction. Carcass weights and adrenal and pituitary weights were recorded at the time of slaughter in both experiments.

Tissue Preparation Techniques.— For studies of total lipids, cholesterol, birefringent material, and ketosteroids, the adrenal tissue slices were fixed in calcium-cadmium formol.[12] For studies of adrenal zona glomerulosa and fasciculata thicknesses and cell-size determinations, tissues were fixed in Helly's and mercury-formol saline [13] mixtures. The latter fixative was used for studies of secretory granules and mitochondrial forms in the zona glomerulosa. All paraffin sections were cut at 7 μ in thickness.

Oil red O [14] was employed as a stain for total lipids. The Nile blue sulfate test was used for neutral and acidic lipids [15] while a modified Schultz's test [16] was employed for cholesterol.

The Ashbel-Seligman test [17] was applied to fixed-frozen tissues for studies of adrenal ketosteroids. An American Optical No. P42AD polarizing microscope was used for studies of birefringent materials. For studies of fluorescence, a No. 5 Spencer research microscope was equipped with a stainless steel first-surface mirror and a quartz lens condenser. The ultraviolet light source was an American Optical microscope lamp fitted with a mercury AH4 bulb and quartz lens, plus Corning glass visible light filters, No. 736 and 760.

A modified Crossman's Mallory triple stain technique [18] was employed routinely on the paraffin tissue sections. Fast green FCF [19] was used as a selective stain for mitochondria.

Studies were made of the relative cell sizes and also of the absolute and relative thicknesses of the zona glomerulosa and the zona fasciculata. A semimicroprojection apparatus was used for the determinations of zone thickness. For the studies of the number of nuclei per unit area (784 μ^2), an ocular net reticule was employed at 440 magnifications in the ocular of a binocular microscope as indicated (table 3).

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TABLE 1—(Experiment 1) Design of Experiment and Quantitative Cytological Estimations * of Oil Red O Stainable Lipids, Birefringent Crystals, and Cholesterol in the Adrenal Zona Glomerulosa

Treatment	Microscopic determination	Animal group ** Group 1 (24 hr. treatment)	Group 2 (36 hr. treatment)	Group 3 (48 hr. treatment)
KCl 2.5% intraven.	Oil Red O	— (65) †	+ (67)†	— (101)†
	Birefringence	—	+	+
	Cholesterol	—	—	—
NaCl 5.0% intraven.	Oil Red O	++ (155)	++ (182)	++++ (242)
	Birefringence	+++	+++	+++++
	Cholesterol	—	+++++	+++++
DCA	Oil Red O	— (180)	++ (270)	+++ (360)
	Birefringence	—	+++	+++
	Cholesterol	—	—	—
Controls	Oil Red O	+	++	—
	Birefringence	++	—	+
	Cholesterol	—	—	—

* The plus and minus signs are based on visual inspection, microscopically, and are to be interpreted as follows: — indicates absence of the substance in question; +++++ indicates that a cell or area was heavily loaded with the substance in question.

** Four animals per group, one animal per treatment.

† Number in parentheses is the total amount (Gm.) of KCl, or the total amount (mg.) of desoxycorticosterone acetate (DCA) administered. *See* "Materials and Methods" for dosage and interval of treatment.

Results of the cytological studies of the pituitary glands will be published elsewhere.

Analyses of the data were done by means of the "t" test.

RESULTS

Oil Red O Stainable Lipids.—Oil red O stainable lipid droplets were found in considerable numbers in the zonae glomerulosae of those animals injected with sodium chloride or DCA (experiment 1), and in those drenched with sodium acetate while water was simultaneously restricted (experiment 2) (tables 1, 2; fig. 1). Either no lipid or very little lipid was present in the zonae glomerulosae of controls in both experiments and in those animals of experiment 2 which were given sodium acetate and water *ad libitum*. The zonae fasciculatae of all treated adrenals as well as

TABLE 2—(Experiment 2) Design of Experiment and Quantitative Cytological Estimations * of Oil Red O Stainable Lipid, Birefringent Crystals, and Cholesterol in the Adrenal Zona Gloomerulosa

Treatment: † Na acetate (Gm.)	Treatment: Water allowed	Av. weight gain or loss (kg.)/ duration of treatment, (days)	Microscopic determination	Animal group ** Group 1	Group 2	Group 3	Group 4
1,245	Restricted	−14/3	Oil Red O	+++	+++++	+++++	+++++
			Cholesterol	+++	++	++	+++
			Birefringence	++++	++++	+++++	++++
1,200	*Ad lib.*	+6/3	Oil Red O	+	+	—	++
			Cholesterol	+	—	—	+
			Birefringence	+	—	—	+
None	*Ad lib.*	+4/3	Oil Red O	—	....	—	....
			Cholesterol	—	....	—	....
			Birefringence	—	....	—	....
None	Restricted	−10/3	Oil Red O	....	—	....	++
			Cholesterol	....	—	....	+
			Birefringence	....	—	....	+

* The plus and minus signs are based on visual inspection, microscopically, and are to be interpreted as follows: — indicates absence of the substance in question; +++++ indicates that a cell or area was heavily loaded with the substance in question.

** Three animals were used per group (e.g., group 1, 2, 3, 4); treatments are replicated with a total of 4 animals per treatment (e.g., line (1), 1,245 Gm. Na acet.) or 2 animals per treatment (e.g., line 4—no treatment with water restricted).

† Dose schedule: 120 Gm. in 600 ml. water 4 times/day, 3 days.

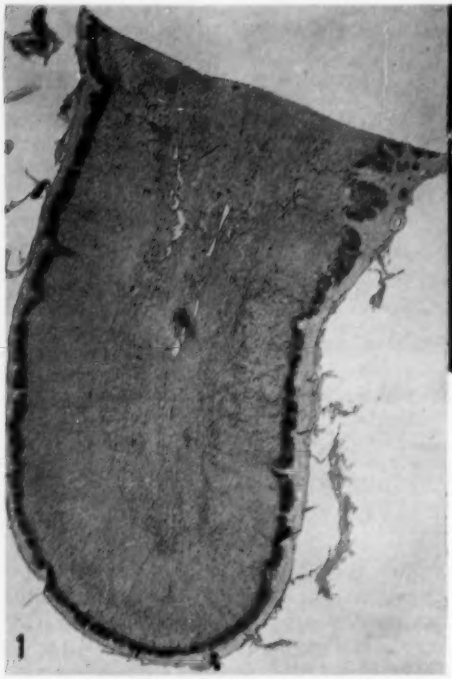

Fig. 1—Adrenal section of a heifer drenched with sodium acetate and restricted on water (table 2); heavy oil red O stained zona glomerulosa, 30μ thick. x 12.

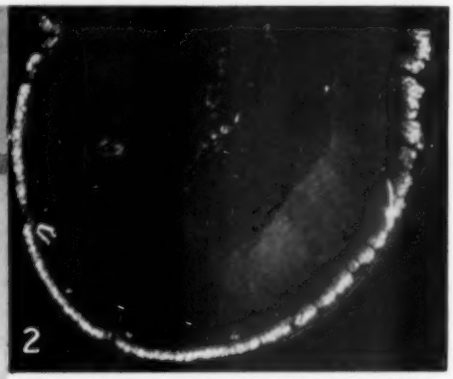

Fig. 2—Adrenal section of a heifer injected intravenously every four hours with 2.5 per cent sodium chloride for 48 hours (table 1); richly birefringent zona glomerulosa. x 12.

control adrenals usually contained no lipid droplets.

Whenever lipid droplets appeared in the zona glomerulosa, they were spherical and 1 to 5 μ in diameter (fig. 3). The majority of lipid droplets measured approximately 1.5 μ. They were most numerous in the centripetal ends of glomerulosa cell clusters. It appeared that the droplets were in positions other than those of the cytoplasmic granules to be described below. Lipid accumulation in adrenal cortical cells ended abruptly at the glomerulosa-fasciculata junction.

In preliminary attempts at characterizing the lipid droplets, the appearance of a pink color with Nile blue sulfate indicated that a significant amount of triglyceride was present.

Birefringent Particles.—The occurrence and number of birefringent particles coincided closely with that of oil red O stained droplets (fig. 1–3; tables 1, 2), in that they were found in the zonae glomerulosae of those animals injected with sodium chloride or DCA and in those drenched with sodium acetate with water simultaneously restricted. The particles appeared in the form of acicular crystals of varying lengths from 4 to 24 μ, but with two principal widths, namely, approximately 0.75 or 1.5 μ (fig. 4). The most frequent types measured 0.75 by 6.0 and 0.75 by 12.0 μ. Studies of fresh-frozen as well as fixed-frozen tissues indicated that the birefringent material was not present in the fresh tissue, but that it slowly crystallized to completion in approximately one week after the initiation of fixation in calcium-cadmium formol. Thus, it was apparent that the crystals were extracellular artifacts.

Cholesterol.—In general, the test for cholesterol was positive in the zonae glomerulosae wherever there was considerable oil red O stainable lipid (tables 1, 2). Thus it is seen that the appearance of a positive cholesterol test, of sudanophilia, and of birefringence followed similar patterns. The positive reaction for cholesterol was limited to the lipid droplets, while birefringent crystals remained observable but uncolored. No cholesterol-positive reactions were observed in the zonae fasciculatae of either experimental or control animals. Myelinated nerves in the adrenal medulla were used as a check of the reaction, since it was found that they showed an intense blue-green color when it developed properly.

Adrenal Ketosteroids.—Tests for adrenal ketosteroids with the Ashbel-Seligman re-

action were inconclusive. The cortical zones of controls as well as treated animals in both experiments usually gave a mild, diffuse positive test while the capsule and medulla were negative. Myelinated nerve fibers and also elastic fibers in the walls of blood vessels, however, were consistently stained a deep purple.

Cytoplasmic, Nonlipid Soluble, Spherical Granules, and Mitochondrial Forms.—In experiment 1, the glomerulosa cells of 2 controls contained a moderate number of orange G-positive spherical granules, while in the third control the cells contained large numbers of these granules. Among them were moderate numbers of short, rod-shaped fuchsinophilic particles which were interpreted to be mitochondria. All of the treated animals were negative for both types of particles. In experiment 2, the zona glomerulosa cells of most animals contained a small to moderate number of secretory granules and small numbers of mitochondria. However, there are fewer of both types of particulates in those animals showing lipid accumulation than in those where the zona glomerulosa was lipid-poor. The mitochondrial forms were too small to be measured. The cytoplasmic inclusions were, as previously described,[19] approximately 1 to 3 μ in diameter. Both cytoplasmic structures reacted positively to the fast green FCF selective stain for mitochondria.

Autofluorescence.—Autofluorescence was found to be associated with the presence of the spherical granules and mitochondrial forms. On close inspection, it was observed that the individual particles were fluorescent rather than the general cytoplasm of the cell. The color of the fluorescence was pale yellow initially but faded or vanished completely in tissues which remained longer than one to two weeks in the fixative.

Mitotic Activity.—Mitotic activity was extremely light and was not observed to be more prevalent in any zone or at any boundary in either treated or control animals in both experiments.

Absolute and Relative Thickness of the Glomerulosa and Fasciculata Zones.—No significant differences due to treatment were found in the glomerulosa or fasciculata zone thicknesses in either experiment (tables 3, 4). The apparent absence of treatment effects, in the authors' opinion, justified the use of these data to record mean and standard error values for the two zones in both experiments for future reference.

While the average values for glomerulosa thickness were nearly identical (176 $\pm$ 7, and 171 $\pm$ 8 μ) in both experiments, there was a significant difference in fasciculata zone thickness between animals in experiments 1 and 2 (823 $\pm$ 36 vs. 1136 $\pm$ 33; $P < 0.01$). It should be noted in this respect that the animals in experiment 2 were larger than those in experiment 1 (136 vs. 58 kg., av. body weights).

Numbers of Glomerulosa and Fasciculata Cells per Unit Area.—These determinations were made for the purpose of establishing whether or not changes occurred in glomerulosa cell size due to treatment effects and whether or not glomerulosa and fasciculata cells were similar or dissimilar in size. For this purpose, the numbers of nuclei were determined per unit area (784 μ^2), since this is equivalent to counting the cells themselves and is more convenient. A visual inspection of these data indicated

TABLE 3—(Experiment 1) Absolute and Relative Thicknesses of Glomerulosa and Fasciculata Zones and Average Numbers of Cell Nuclei per Unit Area

Calf No.	Treatment	Body weight (kg.)	Glom. thickness ** (μ)	Fasc. thickness ** (μ)	Ratio fasc./glom. thickness	Av. No.** of glom. cell nuclei/unit area †	Av. No.** of fasc. cell nuclei/unit area †
1	KCl	79	182	962	5.3	7.9	5.4
2	KCl	42	185	769	4.2	7.5	5.2
3	KCl	51	176	624	3.5	6.0	6.4
4	NaCl	66	139	919	6.6	8.0	4.9
5	NaCl	52	150	820	5.5	7.3	5.4
6	NaCl	66	183	832	4.5	6.8	5.3
7	DCA	71	182	882	4.8	7.5	5.4
8	DCA	65	191	1042	5.5	8.8	5.7
9	DCA	40	136	697	5.1	7.0	5.3
10	Control	67	164	861	5.3	7.9	4.9
11	Control	63	218	815	3.7	7.9	6.2
12	Control	36	203	656	3.2	7.1	4.4
Av. for all animals		58	176$\pm$7*	823$\pm$36	4.7	7.5$\pm$0.21	5.4$\pm$0.15

* Standard error ($S_{\bar{x}}$); ** 40 measurements or counts were made per animal; † 784 μ^2.

clearly that no significant changes occurred in the numbers of cells per unit area in either glomerulosa or fasciculata zones as as a result of treatment. On the basis that there were no significant treatment effects, mean and standard values were computed for all animals in each experiment for future reference (tables 3, 4).

The average number of glomerulosa cells per unit area was found to be significantly greater (*i.e.*, the cells were smaller) than those in the fasciculata zone in both experiments ($P < 0.01$). In addition, it was found that the over-all average for numbers of glomerulosa cell nuclei per unit area was similar for both experiments (av. of 7.5 ± 0.21 and 8.0 ± 0.28, respectively).

In contrast to this, it was found that there was a significant decrease ($P < 0.01$) in numbers of fasciculata cell nuclei per area (4.5 ± 0.16 vs. 5.4 ± 0.15) in experiment 2 as compared to those of experiment 1.

DISCUSSION

An important factor to consider in a discussion of these findings is that the bovine adrenal gland is characteristically lipid-poor.[20, 21] In contrast to this, the outstanding characteristic of the adrenal glands of laboratory animals (e.g., rat, rabbit, dog) is their high content of neutral fat and cholesterol.[22-24] Thus, it is somewhat hazardous to draw parallel conclusions from findings which are similar to those in laboratory animals.

The data presented here concerning lipid accumulation would suggest that the zona glomerulosa was inactivated in those cases where oil red O stainable lipid droplets appeared, since the droplets were coarse. This conclusion is based on an analogy with similar changes noted by Deane [5] and also Bacchus [7] in the zonae glomerulosae of rats which were fed potassium-deficient diets or injected with sodium chloride or with DCA. However, it should be noted that the

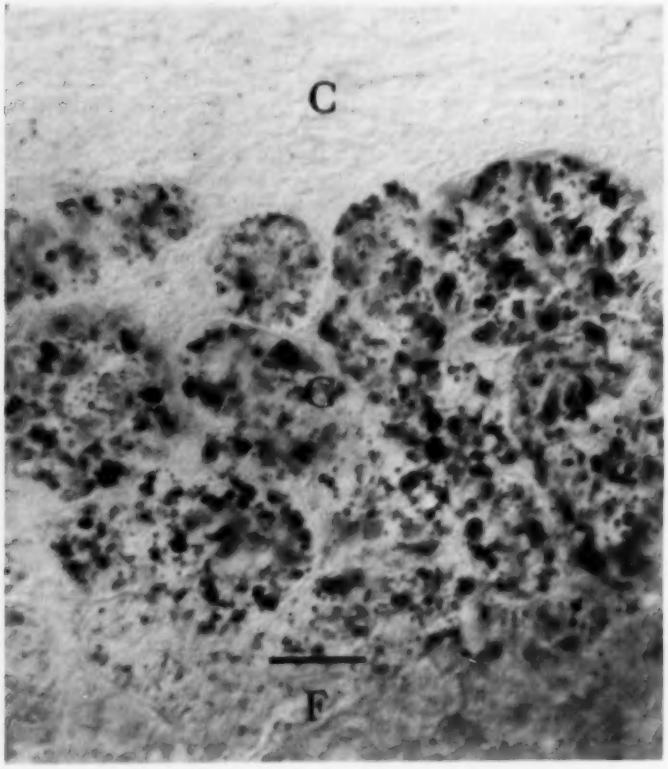

Fig. 3—High-power photomicrograph of oil red O stained lipid droplets from the section shown in figure 1. The most prominent lipid droplet is uniform in size (about 1.5μ) and shape. Note also the larger, somewhat more irregularly shaped lipid droplets. Oil red O stain. x 350.

C = capsule.
G = zona glomerulosa.
F = zona fasciculata.

fine or small fat droplet-type which was found under conditions of hyperactivity by Deane was not found in those animals of our experiments, where the zona glomerulosa would be expected to be stressed as the result of potassium injection. Instead, no lipid droplets of any size were found.

The size and number of lipid droplets produced in the zona glomerulosa cells in both experiments reported here resembled the fatty metamorphosis commonly seen and described in bovine and ovine ketosis. Unfortunately, further comparisons of the experimentally induced cytological changes with those present in the "ketosis complex" can not be made due to a lack of data for the latter conditions.

The pink color obtained with Nile blue sulfate indicated that a significant amount of the lipid in the coarse droplets must be neutral fat, *i.e.*, triglycerides. Although the latter usually are present in abundant quantities in the adrenals of laboratory animals,[22-26] their function is unexplained at present.

The positive cholesterol reaction in lipid droplets of the zona glomerulosa is likewise believed to be an indication of inactivation. This conclusion is based on numerous observations in which it was found that adrenal cholesterol disappeared in laboratory animals under conditions of stress and reappeared after normal environmental conditions resumed.[24-28] The presumption is that cholesterol is a precursor in corticosteroid synthesis. However, the present authors have not been able to obtain a positive test for cholesterol in the adrenal fasciculata zone of either young or mature cattle which were under no apparent stress and even when there was marked adrenal sudanophilia. From the experiments of Hechter,[29] it can be seen that in cattle there are additional possible synthetic pathways to corticosteroids other than through cholesterol. However, it is possible that only in the zona glomerulosa of the bovine adrenal does cholesterol accumulation exceed the minimum quantity required[30] for a positive histochemical reaction.

The sudanophilic and cholesterol-positive droplets of the zona glomerulosa were not positive to the Ashbel-Seligman test for ketosteroids. Rather, the color was spread diffusely in the cytoplasm. Myelinated nerves and elastic fibers constantly showed an intense positive reaction. These unusual results may find an explanation in the work of Karnovsky and Deane[31] in which it was concluded recently, from correlated chemical and histochemical studies, that the Ashbel-Seligman reaction is not specific for ketosteroids. Instead, these authors believe that practically all detectable lipid carbonyl groups of the adrenal cortex are artifacts formed by auto-oxidation of fatty acids.

No interpretations of the nature of the acicular birefringent crystals can be made at present. Yoffey *et al.*[32] failed to establish the identity of these crystals with cholesterol as well as various steroids or neutral lipids.

The spherical granules and mitochondrial forms are of unknown significance as yet. Their irregular appearance in treated as well as in control animals of both experiments makes it impossible to relate them to conditions of either homeostasis, hyperactivity, or hypoactivity. In the authors' experience, they are found only in

TABLE 4—(Experiment 2) Absolute and Relative Thickness of Glomerulosa and Fasciculata Zones and Average Numbers of Cell Nuclei per Unit Area

Calf No.	Treatment	Body weight (kg.)	Glom. thickness ** (μ)	Fasc. thickness ** (μ)	Ratio fasc./glom. thickness	Av. No.** of glom. cell nuclei/ unit area †	Av. No.** of fasc. cell nuclei/ unit area †
1	NaAc—H_2O	147	129	1296	10.0	8.8	3.9
2	NaAc—H_2O	181	144	1119	7.8	8.2	4.0
3	NaAc—H_2O	139	163	1170	7.2	7.9	4.1
4	NaAc—H_2O	118	216	1163	5.4	7.5	4.7
5	NaAc+H_2O	146	173	1187	6.9	8.9	4.8
6	NaAc+H_2O	115	174	1006	5.8	7.1	4.4
7	NaAc+H_2O	135	170	1326	7.8	7.4	4.0
8	NaAc+H_2O	142	185	1057	5.7	8.4	5.1
9	Control+H_2O	119	157	960	6.1	8.5	4.8
10	Control+H_2O	122	151	1237	8.2	7.6	5.0
11	Control—H_2O	145	176	1059	6.0	9.3	5.4
12	Control—H_2O	124	217	1053	4.9	5.8	3.6
Av. for all animals		136	171±8*	1136±33	6.6	8.0±0.28	4.5±0.16

* Standard error ($S_{\bar{x}}$); ** 40 measurements or counts were made per animal; † 784 μ^2.

Fig. 4—This photomicrograph illustrates the size and shape of birefringent crystals of the adrenal section shown in figure 2. x 1,000.

domestic ruminants and always are localized in the zona glomerulosa. The ability of these particulates to fluoresce is of no value in identification since many substances have this property.[15]

Although no quantitative data are available, it is believed that cell size is a reliable criterion of the degree of cellular activity in the adrenal cortex.[23] The findings in the present experiments indicate that there are significantly more zona glomerulosa cells than fasciculata cells per unit area. That is, the glomerulosa cells are considerably smaller than those in the zona fasciculata (tables 3, 4). On this basis, it would seem that the glomerulosa zone is less active than the fasciculata. However, it was not possible under the conditions of the two experiments to cause a significant glomerulosa cellular hypertrophy in those animals which were injected with potassium chloride, or a decrease in cell size in those given sodium chloride or DCA. Likewise, and in contrast to results obtained in rats,[5,7] no increase or decrease in the thickness of the zona glomerulosa could be produced by these treatments. Further studies are needed before interpretations of these contradictory findings can be made.

A significant increase was observed in the thickness of the fasciculata zone for all animals in experiment 2 as compared to those of experiment 1. It is believed, on the basis of general observations in mature cattle,[16] that the changes in the fasciculata zone probably were due to factors associated with a change-over to the ruminant type of digestion seen at this age. It would appear from the present experimental results that the zona glomerulosa is not similarly influenced at this age.

SUMMARY AND CONCLUSIONS

1) In each of two experiments reported here, 12 young Holstein-Friesian heifers, which averaged 58 and 136 kg. in weight, respectively, were used.

Attempts were made to produce lipid accumulation and other cytological changes in the adrenal zonae glomerulosae of these animals similar to those observed in bovine and ovine ketosis and in parturient paresis. The methods used were injections of sodium chloride, potassium chloride, desoxycorticosterone acetate (DCA), or drenches of sodium acetate.

The authors are aware that the number of animals in these experiments is somewhat limited. However, it should be noted that the lipid accumulation response in these and additional unreported experiments was essentially an all-or-none reaction, that the lipid, when present, was uniformly restricted to the glomerulosa zone, and that the calf adrenal is an extremely lipid-poor organ.

2) Oil red O stainable lipids, cholesterol, and acicular birefringent crystals were markedly increased in quantity in those animals of experiment 1, which were injected with sodium chloride or DCA. Similar changes were found in those animals of experiment 2, which were drenched with sodium acetate while water was simultaneously restricted. The acicular crystals were considered to be extracellular precipitation artifacts of material which was present in unusually large amounts in the amorphous state in the living cell.

The total lipid changes produced in these calf experiments (as determined by oil red O) appear to be similar to the cytological picture seen in the zonae glomerulosae of adrenals of cows and sheep with ketosis. However, comparable detailed cytological studies must be made on the adrenals of these ketotic animals before more exact comparisons can be made.

3) With techniques used in these experiments, mitochondrial and secretory granule forms were found to be restricted to the zona glomerulosa. Their presence was irregular in all treated and control groups in both experiments. A pale yellow fluorescence was observed to be associated with these particulates.

4) Mitotic figures were uniformly rare in all animals. When present, they were not localized in any zone or at any boundary.

5) The thickness of the glomerulosa zone was not altered by treatments in either experiment. This zone also was similar in thickness in the adrenals of all animals in both experiments despite the fact that animals of experiment 2 weighed twice as much as those in experiment 1 (136 kg. vs. 58 kg. av.). In contrast, the adrenal fasciculata zone was significantly thicker in those animals of experiment 2 as compared to animals in experiment 1.

6) Cell size in the zona glomerulosa was not altered by treatment in either experiment. The number of nuclei per unit area was significantly greater (*i.e.*, the cells were smaller) in the zona glomerulosa as contrasted to the zona fasciculata in both experiments.

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Observations on Collagen, Vitamin A, and Ascorbic Acid in Bovine Hyperkeratosis

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AMONG the more striking lesions of bovine hyperkeratosis are the pathological changes which take place in the skin.[1,2] It is reasonable to suspect that associated with hyperkeratosis, thickening, and loss of elasticity there are also alterations in the chemical makeup of the skin. One of the components of the skin which might be affected is collagen. Therefore, both qualitative and quantitative observations on this important structural protein should be of interest. The low level of vitamin A found in the blood of animals with bovine hyperkeratosis,[4] along with a disturbed ascorbic acid metabolism,[8,11] is also of importance. The respective roles of these vitamins in the development of the epithelial and connective tissues and in maintaining the normal physiology of these tissues are well established.[17-19]

In this communication, the results of quantitative observations on skin collagen in normal calves and in calves with bovine hyperkeratosis are reported. Data are presented which indicate that the physical changes in the skin are accompanied by chemical alterations. Also reported are observations on the effects of the administration of chlorinated naphthalene on plasma levels of carotene, vitamin A, and total ascorbic acid.

MATERIALS AND METHODS

The various experimental animals and the type of treatment given are described (table 1). The calves numbered 1 through 8 were normal, untreated animals 1 to 8 months old. Calves 62 and L65 were also untreated animals 4 to 5 months old, which were obtained at a later date to provide additional normal skin samples. Animals 10, 11, 12, and 13 were yearling heifers from a herd affected with a field outbreak of bovine hyperkeratosis. Calves 14 and 15 received alfalfa pellets which had been responsible for a field outbreak of bovine hyperkeratosis.[13] Analyses of the pellets * indicated that they contained a mixture of chlorinated naphthalenes, probably the tetra- and pentachloro compounds. The exact amount of each component could not be accurately determined. A commercial preparation containing penta- and hexachloronaphthalenes (62 % chlorine) was administered to calves 16, 17, 18, and 19. Calf 20 and cows 21, 22, 23, and 24 were given a purified preparation of pentachloronaphthalene.** The cows were all pregnant. All of the experimental animals, with the exception of those affected by the field outbreak, were fed good quality alfalfa hay and a rolled oats supplement as a basic ration.

Analyses for carotene and vitamin A were done by the method of Kimble.[9] Total plasma ascorbic acid was determined by the method of Roe and Kuether.[16] Collagen was determined by the method of Lowry, Gilligan, and Katersky.[10] Skin biopsies were taken from areas of the neck and withers where the lesions were most marked. The samples were prepared for analysis by removing the hair with a barium sulfide depilatory and by removing any loose, keratinized material from the surface with a stiff fiber brush. Portions of from 2 to 4 Gm. were then reduced to a smooth paste in a Waring blendor with about 20 ml. of 0.1 N sodium hydroxide. The blendor was kept at 4 C. during this operation. Analyses were done in duplicate on fresh samples and on samples which had been stored at −60 C. for periods up to 250 days. A third portion of each skin sample was placed in 10 per cent formalin (pH 7.0) and examined for collagen after storage ranging from one to 492 days.

RESULTS

The collagen content of skin from 6 normal calves ranged from 22 to 30 Gm. per 100 Gm. of skin. Similar values were obtained for fresh, frozen, and formolized samples. The values for collagen in fresh or frozen samples from animals with bovine hyperkeratosis ranged from 2 Gm. to 14 Gm. per 100 Gm. of skin, whereas values of from 19 to 30 Gm. per 100 Gm. were

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* Infrared and ultraviolet analyses of extracts from the pellets were made by L. V. Curtin of the Buckeye Cotton Oil Company. The extracts were also analyzed colorimetrically by R. W. Engel of the Department of Biochemistry and Nutrition, Virginia Agricultural Experiment Station, Blacksburg.

** The pentachloronaphthalene used in these experiments was supplied by Dr. Wilson Bell of the Virginia Agricultural Experiment Station, Virginia Polytechnic Institute, Blacksburg.

TABLE 1—Data on Experimental Animals and Treatment Which They Received

Animal (No.)	Age	Material	Amount	Amt./lb. body weight	Days administered
1–8	1–8 mo.	None	.....	.....	.....
62, L65	4–5 mo.	None	.....	.....	.....
9	5 yr.	None	.....	.....	.....
10–13	1 yr.	Field outbreak	.....	.....	.....
14	5 mo.	Toxic Pellets	29 lb.	.....	52
15	6 mo.	Toxic pellets	58 lb.	.....	58
16	6 mo.	Chlorinated naphthalenes *	1.00 Gm.	2.52 mg.	6
17	6 mo.	Chlorinated naphthalenes	0.816 Gm.	2.52 mg.	6
18	6 mo.	Chlorinated naphthalenes	0.816 Gm.	2.52 mg.	6
19	6 mo.	Chlorinated naphthalenes	0.756 Gm.	2.52 mg.	6
20	4 mo.	Pentachloronaphthalene *	1.48 Gm.	5.00 mg.	7
21	4–5 yr.	Pentachloronaphthalene *	3.60 Gm.	5.00 mg.	6
22	4–5 yr.	Pentachloronaphthalene	2.70 Gm.	2.50 mg.	6
23	4–5 yr.	Pentachloronaphthalene	1.05 Gm.	1.25 mg.	6
24	4–5 yr.	Pentachloronaphthalene	0.45 Gm.	0.63 mg.	6

(*) Given *per os* by capsule as a 2 % solution in cottonseed oil.

found for the portions treated with formalin (table 2). Low collagen values were also observed in samples of skin from 2 apparently normal animals (calves 1 and 8). Formolized samples from these calves yielded normal collagen values.

The action of formalin on the abnormal skins took place within 24 hours and prolonged exposure to the chemical was not necessary to induce the change to higher collagen values. For example, fresh skin from calf 17 contained 5 Gm. of collagen per 100 Gm. but when treated with formalin for 24 hours, 48 hours, 10 days, and 246 days the values increased to 29, 26, 28, and 27 Gm. per 100 Gm., respectively.

TABLE 2 — A Comparison of the Observed Collagen Content of Skin, Fresh and Formalin-Treated (24 hr. or Longer), from Normal Animals and from Animals with Bovine Hyperkeratosis

Animal (No.)	Skin condition	Treatment of sample	Collagen Gm./100 Gm. skin
1	Clinically normal	Fresh	1
		Formalin	25
2	Normal	Fresh	22
		Formalin	26
3	Normal	Fresh	23
		Formalin	23
4	Normal	Fresh	30
		Formalin	29
5	Normal	Fresh	29
		Formalin	29
8	Clinically normal	Fresh	1
		Formalin	25
62	Normal	Fresh	24
		.....	....
L65	Normal	Fresh	25
		.....	....
9	Dermatosis	Fresh	3
		Formalin	27
10	Hyperkeratosis	Fresh	6
		Formalin	27
11	Hyperkeratosis	Fresh	2
		Formalin	26
12	Hyperkeratosis	Fresh	10
		Formalin	30
13	Clinically normal	Fresh	5
		.....	....
14	Hyperkeratosis	Fresh	2
		Formalin	21
15	Hyperkeratosis	Fresh	3
		Formalin	19
17	Hyperkeratosis	Fresh	5
		Formalin	27
19	Hyperkeratosis	Fresh	14
		Formalin	23
20	Hyperkeratosis	Fresh	8
		Formalin	21

The plasma ascorbic acid levels of calves 16, 17, 18, and 19 before and after poisoning with chlorinated naphthalenes are shown (fig. 1). The plasma ascorbic acid levels dropped sharply after poisoning and were lower than those of the control animals thereafter. These values represent total plasma ascorbic acid which includes dehydroascorbic acid. Consequently, they were higher than the values reported by other workers[8,11] for reduced ascorbic acid only.

The effect of the chlorinated naphthalenes on the plasma vitamin A levels of calves 16, 17, 18, and 19 (fig. 2) illustrates the response of different animals of similar age and breed to a uniform dose of the toxic material. Calf 17, for example, had a plasma vitamin A level of 18.8 μg. per 100 ml. when the poisoning was started. The value had dropped to 8.7 μg. per 100 ml. after seven days, a decrease of 54 per cent. The initial plasma vitamin A level of calf 18 was 28.5 μg. per 100 ml., and after seven days it had dropped to 3.1 μg. per 100 ml. for a decrease of 89 per cent. Calf 17 died 172 days after poisoning began and calf 18 died after 29 days.

The changes in plasma carotene and vitamin A of calf 20 after poisoning with pen-

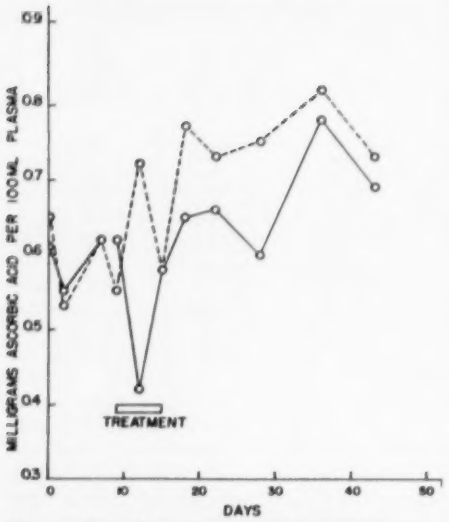

Fig. 1—The effect of chlorinated naphthalenes on plasma ascorbic acid levels. The broken line represents the average values of 2 untreated animals (calves 6 and 7). The solid line represents the average values of 4 treated animals (calves 16, 17, 18 and 19) each of which received a total of 2.52 mg. per pound of body weight of a mixture of penta- and hexachloronaphthalenes.

tachloronaphthalene are shown (fig. 3). At the start of the experiment, plasma vitamin A was 21.0 μg. per 100 ml. Seven days after the beginning of poisoning, plasma vitamin A was 7.1 μg. per 100 ml. This was a 67 per cent drop in vitamin A. The initial level was not regained during the experimental period. The decrease in carotene was not as marked and the initial level was regained after 44 days.

The response of cows 21, 22, 23, and 24 to pentachloronaphthalene poisoning is illustrated (fig. 4). All 4 animals showed the expected decrease in plasma vitamin A but there was a comparatively small decrease in plasma carotene. The single curve for carotene (fig. 4) is for cow 21 which received the highest amount of poison and is representative of the corresponding curves for the other 3 cows. Cow 22 aborted 21 days after administration of the toxic material; however, it was not possible to determine whether this was a direct consequence of the poisoning.

DISCUSSION

The collagen content of skin from animals with bovine hyperkeratosis is markedly lower than in normal animals. The reaction of the abnormal skins in formalin suggests that in bovine hyperkeratosis there is an alteration in the process leading to the deposition of collagen from its precursors. Wolbach [18, 19] favors the view that collagen originates as an amorphous ground substance secreted by fibroblasts. In the presence of ascorbic acid this ground substance is supposed to "jell," thus being converted to collagen fibers. There is considerable evidence for soluble precursors to collagen.[6, 14] Recently, Harkness and his associates [5] presented evidence that an alkali soluble fraction from connective tissue is almost certainly a true precursor of collagen.

The evidence for "ground substances" or precursors of true collagen suggests a possible explanation for the differences in collagen content observed in fresh and formalin-treated abnormal skins. The collagen-determination method used is based upon the conversion of insoluble collagen to gelatin. As a preliminary step to the gelatin conversion, the sample is treated for 12 to 18 hours with 0.1 N sodium hydroxide. It is then extracted with a mixture of alcohol and ether. The supernatant liquids are discarded at each of these steps so that an alkali soluble frac-

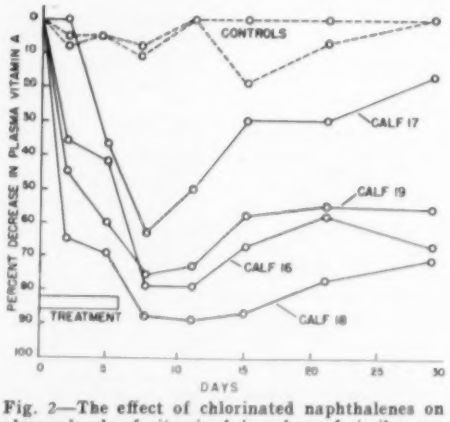

Fig. 2—The effect of chlorinated naphthalenes on plasma levels of vitamin A in calves of similar age and breed. The solid lines represent the changes in plasma vitamin A of calves 16, 17, 18 and 19, each of which received a total of 2.52 mg. per pound of body weight of a mixture of penta- and hexachloronaphthalenes. The broken lines represent the changes in plasma vitamin A for calves 6 and 7 which were untreated.

tion not yet converted to collagen by the damaged skin would be lost. Axelrod and Martin [1] noted a loss of collagenous material in thermally damaged rat skin during preliminary treatment with 0.9 per cent saline, ethanol, ether, and 0.1 N sodium hydroxide.

Gustavson,[3] in reviewing the chemical aspects of tanning, states that the action of a tanning agent, such as formalin, on collagen is to make it less soluble in water and improve its stability. The tanning agent accomplishes this by supplying stable cross links which bind the protein molecules together. It seems reasonable that formalin could react with precursors of collagen to render them less soluble in alkaline reagents or to bring about their aggregation into collagen or collagen-like material. Either collagenous material or a chemically similar protein fraction, which could be solubilized in the gelatin conversion step of the analytical procedure of Lowry *et al.*,[10] would then be measured as collagen.

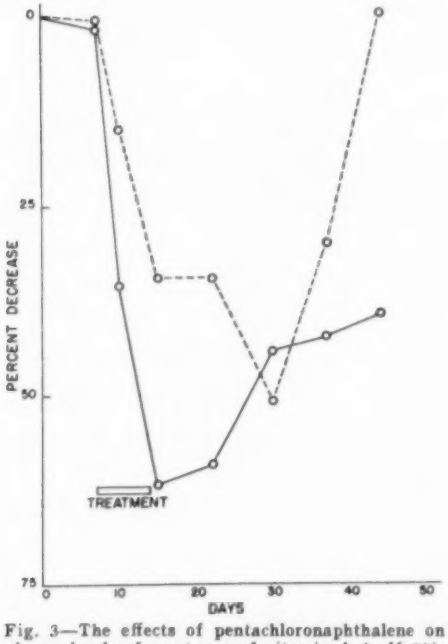

Fig. 3—The effects of pentachloronaphthalene on plasma levels of carotene and vitamin A (calf 20). The solid line represents vitamin A, the broken line carotene. The animal received a total of 5 mg. of pentachloronaphthalene per pound of body weight. Note that the initial carotene level was regained after 44 days while the vitamin A level remained low.

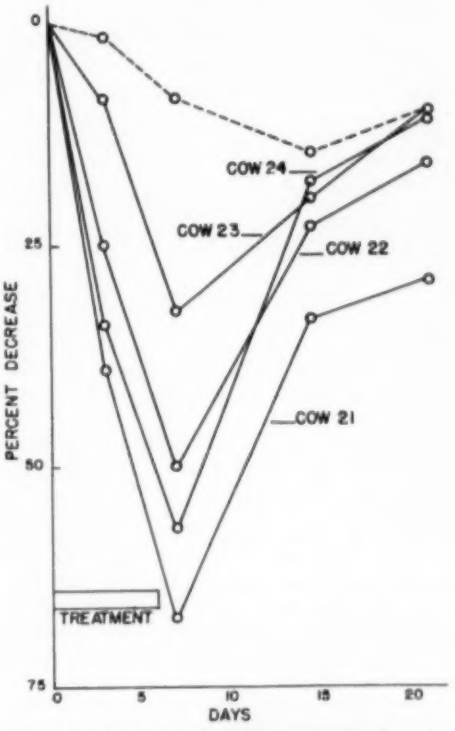

Fig. 4—The effects of various amounts of pentachloronaphthalene on the plasma carotene and vitamin A levels of cows. The solid lines represent the percentage decrease in plasma vitamin A. The broken line shows the percentage decrease in plasma carotene for cow 21 and is representative of the changes in plasma carotene for the other cows. The animals received the following total amounts of pentachloronaphthalene per pound of body weight: Cow 21, 5.00 mg.; cow 22, 2.50 mg.; cow 23, 1.25 mg.; cow 24, 0.63 mg. Cow 22 aborted on the twenty-first day. Note that the depression of plasma vitamin A levels correlates directly with the amount of toxic material administered.

The apparent increase in collagen after formalin treatment of skin from cow 9 indicates that abnormal skin conditions, other than bovine hyperkeratosis, may alter proteins so that they give the anomalous reaction with formalin. This animal had a chronic dermatosis which was not hyperkeratotic in nature. Her plasma carotene and vitamin A levels were normal and she was otherwise apparently normal. No explanation of the anomalous collagen values observed for calves 1 and 8 is possible at present. Clinically, these animals appeared to be normal. Age was probably not a

significant factor since calf 1 was 1 month old and calf 8 was 8 months old.

Ascorbic acid is essential for the formation of collagen.[15] Furthermore, it has been shown [2] that in cattle the level of plasma ascorbic acid is contingent upon the level of vitamin A. This is particularly so when the plasma vitamin A values are below 10 μg. per 100 ml. In avitaminosis A in rats, the histological changes in teeth and muscle parallel the decreasing blood ascorbic acid level. The organs which synthesize vitamin C may be damaged by a deficiency of vitamin A. There is also evidence [15] of a synergistic relation between vitamin E (tocopherol) and ascorbic acid. Hove [7] found a marked decrease of plasma tocopherol levels in animals with bovine hyperkeratosis.

In a previous report [13] from this laboratory on bovine hyperkeratosis, it was indicated that plasma levels of vitamin A in affected animals were similar to levels in unaffected animals. The values reported at that time were in error because of improper analytical procedures. With correct procedure, we have since found low values for vitamin A in bovine hyperkeratosis in agreement with the results of other workers.

The response of calf 20 to poisoning with pentachloronaphthalene (fig. 3) suggests a possible interference with the metabolic systems involved in the conversion of carotene to vitamin A, since the plasma level of carotene returned to normal while vitamin A did not. This interference was suggested earlier by Hansel and Olafson.[4] The correlation between dosage of pentachloronaphthalene and decrease in plasma vitamin A is illustrated by the data from cows 21, 22, 23, and 24 (fig. 4). The rapid return of plasma vitamin A and carotene to initial levels in the older animals suggests that they may be less susceptible to the action of chlorinated naphthalenes than calves.

SUMMARY

Calves with bovine hyperkeratosis had decreased amounts of collagen in their skins. Plasma levels of vitamin A and ascorbic acid were depressed after poisoning with chlorinated naphthalene. The available evidence indicates that in bovine hyperkeratosis the pathological changes in epithelial and connective tissues may be due to these changes in vitamin A and ascorbic acid metabolism.

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Vitamin A Deficiency and Urolithiasis in Range Cattle

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UROLITHIASIS with urethral occlusion is the cause of considerable economic loss from the deaths of a large number of range steers each year.[1] The fact that the losses occur almost exclusively during the mid-winter months [2, 3] suggests that the disease may be associated with a vitamin A deficiency, developing after the steers have become depleted of their vitamin A reserves while they are on forage which is nearly devoid [4] of carotene. It has been suggested that pathological changes in the urinary tract resulting from vitamin A deficiency may contribute to the formation of calculi.

That a vitamin A deficiency may lead, under certain circumstances, to urolithiasis in rats has been demonstrated.[5-11] Extension of this type of study to other species has not resulted in such clear-cut results. This is especially true of the domestic ruminants, cattle, sheep, and goats. In some cases, a high incidence of urolithiasis appears to be associated with rations which are supposedly low in carotene and in vitamin A.[12-18] However, in relatively few of these reports [14-16, 18] has the existence of a vitamin A deficiency been confirmed by a blood analysis or a clinical examination. On the other hand, there appear in the literature a number of reports of intentional vitamin A deficiency in cattle, sheep, and goats [15, 19-27] in which urolithiasis was not recorded. Unfortunately, in only a few of these reports [15, 19, 22, 24, 27] is it indicated that a postmortem examination was made, so that the possibility of subclinical calculosis is not ruled out in most of these studies.

A number of reports have also appeared [20, 28-43] in which a high incidence of urolithiasis is associated with what appears to be an adequate vitamin A intake. However, in none of these reports was the adequacy of the vitamin A status of the animals confirmed by blood analysis or otherwise.

Urolithiasis differs in range and feedlot steers in that the calculi from range steers are largely composed of silica (SiO_2)[28] while those occurring in feedlot animals are more commonly phosphates and carbonates.[29-45]

In an effort to determine the relationship of vitamin A deficiency to urolithiasis in range cattle, three types of evidence have been accumulated and are reported herein. First, blood plasma vitamin A levels have been determined in clinical urolithic cases and in unaffected animals in herds where calculosis is occurring. Second, reports from field investigations, letters from cattle ranchers, and data from questionnaires sent to cattle producers have been evaluated with regard to the probable presence or absence of adequate carotene in the rations of the affected animals. Third, a vitamin A deficiency was induced experimentally in a group of steer calves on a ration simulating range forage, and appropriate urine examinations were made to detect any tendency toward urolithiasis.

VITAMIN A IN BLOOD PLASMA

The blood plasma from 46 calves with clinical urolithiasis was analyzed for vitamin A. The mean value was 15.3 μg. per 100 ml., with a standard deviation of 7.3. Of these samples, 26 (57 %) were below 16 μg./100 ml. and 20 (43 %) were 16 or above. The value of 16 μg./100 ml. is the critical plasma vitamin A level for nonreproducing cattle, according to Davis and Madsen.[27]

The blood plasma from 112 calves not suffering from clinical urolithiasis, but from herds in which cases of clinical urolithiasis were occurring, contained an average of 18.6 μg./100 ml., with a standard deviation of 6.4. Of these 112 plasma samples, 40 (36 %) were below 16 μg./100 ml., and 72 (64 %) were 16 and over.

The difference between the means of plasma vitamin A levels of the affected calves and the nonaffected calves is statistically significant at the 1 per cent level.

Unfortunately the authors do not have the results of the vitamin A analyses of a sufficient number of plasma samples from calves in comparable noncalculous herds to provide figures for comparison with the above.

Although analysis of many of the plasma samples from animals with clinical cases of urolithiasis indicated a possible vitamin A deficiency, there were also many which had an adequate level of this vitamin. Among the 46 samples taken from animals with occluding urolithiasis, there were nine between 16 and 19 μg./100 ml., eight between 20 and 29, and three above 30. At times, the authors have thought that occluding urolithiasis, particularly cases of long duration, may have

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caused a lowered plasma vitamin A, but insufficient data are available to support or refute this hypothesis.

FIELD OBSERVATIONS

Of the letters received at this laboratory during the past few years concerning urinary calculi in range steers, 12 discussed the feed which the animals had been getting. Of these, 11 stated that the animals were eating good green feed. Only one said that the feed was not green.

Six field investigations of herds with calculosis have been made by our staff. In five of these, the feed was found to be good green hay or pasture. The sixth described the hay as of fair quality.

For several years a study of urolithiasis in Meagher and neighboring counties has been made by means of questionnaires. Among the questions asked, has been one concerning the quality of the pasture or hay being fed to the animals. Ninety replies to this question have been received from ranches experiencing difficulty with urinary calculi. Of these, 70 described the hay as good or excellent, 12 as fair, and eight as poor. Of six replies received from ranches without calculosis, five described their hay as good and one as fair.

EXPERIMENTAL VITAMIN A DEFICIENCY

Purpose.—This experiment was to determine whether a vitamin A deficiency in steer calves fed grass hay results in urinary changes which might be associated with urolithiasis.

Plan.—In each of the two winters prior to the one in which this experiment was performed, attempts had been made to produce a vitamin A deficiency in range calves by placing them on a bleached hay ration at the time of weaning. These calves failed to become deficient by December, which is the time field cases of urolithiasis usually appear. For this reason it was decided to start depleting the animals in midsummer, while the calves were still nursing.

A group of range cows with steer calves at their sides was to be placed on a carotene-free hay ration and divided into two lots. One lot was to be supplemented with vitamin A and the other was to receive only the carotene-deficient ration. At weaning time the cows were to be sold and the calves retained, maintaining the two lots on vitamin A-deficient and A-supplemented rations, as were their dams.

The development of the vitamin A deficiency was to be followed by blood plasma and liver biopsy analyses.

The urine from each of the calves was to be periodically examined physically and chemically for evidence of any variation which might be associated with calculus formation.

Materials.—Animals.—Twenty-two range Hereford cows with bull calves at their sides were received on June 30, 1953. The calves were castrated four days later.

Feeds.—The analyses of feeds used follows:

1951 carotene-low hay—huntley mixture (red top 53%, blue grass 45%) deliberately bleached in swath and windrow for four weeks. Analysis (1954): protein 8.1%, phosphorus 0.18%, carotene 0.0 μg. per gram.

1952 carotene-low hay—similar to above. Bleached five weeks. Analysis: protein 9.1%, phosphorus 0.18%, carotene 2.0 μg. per gram.

Nikles hay—bleached four weeks. Analysis: protein 4.8%, phosphorus 0.11%, carotene 1.5 μg. per gram.

Smart hay—bleached six weeks. Analysis: protein 7.2%, phosphorus 0.13%, carotene 2.8 μg. per gram.

Dairy hay—not bleached. Analysis: protein 9.4%, phosphorus 0.27%, carotene 23.0 μg. per gram.

Mandeville straw—analysis: protein 1.5%, phosphorus 0.024%, carotene 0.0 μg. per gram.

Ground barley—analysis: carotene 0.0 μg. per gram.

Vitamin A concentrate—Pfizer vitamin A acetate in gelatin, 10,000 I. U. per gram.

Mixed feed—2 parts barley with 1 part soy bean oil meal.

Water.—All calves had continual access to water. Analysis: total solids 266 p.p.m. (parts per million), silica 53 p.p.m., sulfate 9 p.p.m., phosphate nil, magnesium 13 p.p.m., chloride 2 p.p.m., calcium 52 p.p.m., carbonate 135 p.p.m.

Methods.—Blood Sampling.—Fifty milliliters of blood was drawn from the jugular vein into a centrifuge tube containing 1 ml. of saturated sodium citrate solution.

Liver Biopsies.—The biopsies were taken by the procedure of Seghetti and Marsh.[54]

Urine Collections.—The collections were made by using a harness consisting of a broad canvas girdle equipped with straps for fastening it around the abdomen of the calf. Inserted in an opening in this girdle was a broad, shallow, stainless steel funnel which drained into a pint fruit jar screwed to its lower side. The prepuce and the surrounding area were kept clipped and were washed before each collection. Each urine sample was stirred and divided into two portions. One portion was preserved with a few drops of formaldehyde solution and the other was refrigerated if its examination could not be completed the same day.

Plasma Phosphorus Determinations.—The determinations were made by the method of Fiske and Subbarow,[55] modified by using 2,4-diaminophenol instead of 1,2,4-aminonaphtholsulfonic acid as a reducing agent.[56]

Plasma Carotene and Vitamin A Determinations.—These determinations were made by a slight modification of the method of Boyer, Phillips, and Smith,[58] in which the carotene is precipitated before determining vitamin A. When carotene levels were low, the carotene precipitation step was omitted, and in its place a correction for the Carr-Price color due to carotene was made.

Liver Vitamin A Determinations.—These determinations were made by saponifying the liver

TABLE 1—Feeding Schedule of Cows and Calves Supplemented with, and Deficient in, Vitamin A

	Supplemented (lot 1)						Deficient (lot 2)			
	Cows			Calves			Cows		Calves	
Date	Roughage	Concentrate	Units of vit. A	Roughage	Concentrate	Units of vit. A	Roughage	Concentrate	Roughage	Concentrate
7/1	1951	.....	.....	1951	.....	.....	1951	.....	1951	.....
7/16	1951	2 lb. barley	.....	1951	.....	.....	1951	2 lb. barley	1951	.....
7/23	1951	.....	.....	1951	.....	.....	1951	.....	.....	.....
7/24	1951	1 lb. barley	.....	1951	.....	.....	1951	1 lb. barley	.....	.....
7/25	1951	2 lb. barley	.....	1951	.....	.....	1951	2 lb. barley	.....	.....
8/8	1951	2 lb. barley	400,000	1951	.....	.....	1951	2 lb. barley	.....	.....
8/10	1952	2 lb. barley	400,000	1951	.....	.....	1952	2 lb. barley	.....	.....
8/27	Nickles	2 lb. barley	400,000	Nickles	.....	.....	Nickles	2 lb. barley	Nickles	.....
9/2	Nickles	2 lb. barley	400,000	Nickles	½ lb. barley	20,000	Nickles	2 lb. barley	Nickles	½ lb. barley
9/3	Dairy	2 lb. barley	400,000	Dairy	½ lb. barley	20,000	Nickles	2 lb. barley	Nickles	½ lb. barley
9/4	Dairy	2 lb. barley	.....	Dairy	½ lb. barley	.....	Nickles	2 lb. barley	Nickles	½ lb. barley
9/16	Dairy	2 lb. barley	.....	Dairy	½ lb. barley	.....	Smart	2 lb. barley	Smart	½ lb. barley
9/27	Nickles	2 lb. barley	400,000	Nickles	½ lb. barley	20,000	Nickles	2 lb. barley	Nickles	½ lb. barley
10/10		(Cows removed)		Nickles	1 lb. barley	20,000		(Cows removed)	Nickles	1 lb. barley
10/14				Nickles	1 lb. mix	20,000			Nickles	1 lb. mix
10/17				Nickles & straw	1 lb. mix	20,000			Nickles & straw	1 lb. mix
10/18				Nickles & straw	1.5 lb. mix	20,000			Nickles & straw	1.5 lb. mix
10/25				Nickles & straw	2 lb. mix	20,000			Nickles & straw	2 lb. mix
12/15				Nickles & straw	3 lb. mix	20,000			Nickles & straw	3 lb. mix
12/22				Straw	3 lb. mix	20,000			Straw	3 lb. mix

2/27 One of the deficient calves had access to vitamin A-supplemented grain for 3 days.
5/10 Eight of the 11 steers in lot 2 broke out and fed on green grass for 30 to 60 minutes.
5/12 All animals placed on bleached grass hay, alfalfa, and grain.
5/18 All animals removed to pasture.

with alcoholic potassium hydroxide, extracting the vitamin A with hexane, evaporating the solvent, taking up the residue with chloroform, and applying the Carr-Price antimony trichloride reagent.[30]

Urinary Silicon Determinations.—These were performed by an unpublished procedure[29] in which the urine was fused with a sodium nitrate-sodium carbonate mixture. The fused mass was dissolved and treated with an acid molybdate solution to form yellow silico-molybdates and phospho-molybdates. The latter were decolorized by the addition of tartaric acid, leaving the former to be measured spectrophotometrically.

Urine Specific Gravities.—These were determined with an ordinary urine hydrometer.

Urine Conductivities.—These were made with a Solu-Bridge after diluting the urine 1:50 with distilled water.

Urine Surface Tensions.—These were made with the DeNouy tensiometer.

Urine pH.—This was determined with a glass electrode pH meter.

Urine Turbidity.—This was determined by measuring in a photoelectric spectrophotometer the optical density of a sample of urine against a blank of the same urine centrifuged to remove suspended material. A wave length of 650 mμ was used, since this is the color at which urinary pigments show a minimum of absorption.

Urine Viscosity.—This was measured in Ostwald viscosimeters at 30 C., using filtered urine. In one experiment, urine samples were all diluted to the same specific gravity (1.010) before measuring the viscosity.

Microscopic Examination of Urinary Sediments.—This examination was made by placing a drop of the urine on a slide, covering it, and viewing it under the microscope. Descriptions of the individual particles are inadequate due to the fact that the urinary sediments from bovine urine have not been adequately described in the literature, and they are mostly different from those found in human urine.

Examination of Dried Films of Urine.—The procedure of Puntriano[26] was followed. A thin uniform smear of the well-mixed urine was made on a clean slide by means of an applicator stick. The slide was immediately set vertically in an oven at 75 C. for a few minutes to dry the film. The dried film was examined under the microscope by incident light by means of a Leitz ultropak objective. As the urine dried, the solids separated as crystals and amorphous material. The results of this examination are expressed on a numerical scale, as follows:

No. 6 represents complete colloid protection, so that no crystals are visible; only a smooth, amorphous film.

No. 5 shows a few small separated crystals with their surfaces completely coated with amorphous film.

No. 4 consists of complete coverage of the smear with small, feathery crystals. These crystals, however, fail to demonstrate good reflections from their surfaces, so their borders give a soft diffusion to the reflected light.

No. 3 shows larger crystals with many brilliantly reflecting surfaces, but there is still some evidence of coating film.

No. 2 and

No. 1 represent larger and more perfect crystals with greater degrees of reflection from their surfaces.

Protective Colloid Determination.—The following procedure[28] was developed for determining the ability of a urine sample to protect a gold sol from precipitation by an electrolyte. The gold sol was prepared by Zsigmondy's formaldehyde reduction method,[28] and stored in a polyethylene bottle. Six tubes, each containing 2.0 ml. of gold sol, were treated with 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 ml. of dialyzed urine, respectively. Each was then treated with 0.20 ml. of 10 per cent sodium chloride solution and mixed. The change from red to blue was recorded as −, +, or ++ according to whether there was a complete change (no protection), a partial change (partial protection), or no change (complete protection), respectively. The reciprocal of five times the volume of urine giving a + reaction was taken as the amount of urine protecting 10 ml. of gold sol from precipitation.

Night Blindness.—This was evaluated by running the steers at dusk through a simple maze of panels and observing their ability to avoid the panels.

Weights of the Calves.—The weights were determined in December, April, and March.

Experimental.—The feeding schedule of all the animals is given (table 1).

Blood samples were taken on: July 3, 1953 (calves only), July 7 (cows only), August 4, September 1, September 29, October 27, November 24, December 21, January 19, 1954, February 16, March 16, April 13, and May 11.

Liver biopsies were made on November 24 and May 5.

Urine collections were made on November 10–12, December 8–10, February 2–3, February 23, March 8–9, and April 19–20.

The calves were tested for night blindness on the evening of May 11, and the following day their eyes were examined for the presence of lesions.

The calves were weighed on December 2, December 31, April 15, and May 12.

RESULTS AND DISCUSSION

The data accumulated with regard to the status of the cows and calves during the experiment are presented (table 2). Because the amount of phosphorus supplied the growing calves by their ration was thought to be near the lower limit of sufficiency, blood phosphorus determinations were made periodically. The table

shows that at no time was there hypophosphatemia.

The plasma carotene levels of both the cows and their calves fell rapidly after they were placed on the low carotene ration. The plasma vitamin A level of the cows rose during July. Thereafter it remained high in the supplemented cows but fell somewhat in the unsupplemented ones. The plasma vitamin A of the calves also rose during the early part of the experiment, but in the unsupplemented calves it had fallen by November 24 to 16 μg./100 ml., and it remained below that level for

TABLE 2—Blood Plasma Phosphorus, Carotene, and Vitamin A of Vitamin A-Supplemented and A-Deficient Cows and Calves, and Liver Vitamin A of Calves

Date	Supplemented		Deficient		t-value	No. of animals	Significance
	Mean	s.d.	Mean	s.d.			
BLOOD PLASMA INORGANIC PHOSPHORUS (mg./100 ml.)—COWS							
8/ 4/53	4.8	0.7	4.6	0.9	0.75	20	
9/ 1/53	4.2	0.8	6.2	1.1	4.75	19	**
9/29/53	4.6	0.7	5.0	0.6	1.28	20	
BLOOD PLASMA INORGANIC PHOSPHORUS (mg./100 ml.)—CALVES							
8/ 4/53	7.4	0.4	7.3	0.7	0.60	21	
9/ 1/53	7.4	0.8	7.6	0.9	0.43	21	
9/29/53	7.2	0.6	7.3	0.7	0.04	21	
10/27/53	7.0	0.5	7.3	0.7	1.07	21	
11/25/53	7.3	0.5	7.6	0.6	1.21	21	
12/21/53	8.2	0.8	8.0	0.8	0.52	21	
1/19/54	8.0	0.7	8.0	0.7	0.07	21	
2/16/54	7.6	0.7	7.5	0.6	0.53	20	
3/16/54	7.7	0.8	7.8	0.9	0.14	20	
4/13/54	7.7	0.6	7.7	0.6	0.08	20	
5/11/54	7.4	0.4	7.2	0.7	0.54	20	
BLOOD PLASMA CAROTENE (μg./100 ml.)—COWS							
7/ 7/53	605.0	188.0	698.0	198.0	1.07	19	
8/14/53	98.0	26.0	129.0	41.0	2.03	20	
9/ 1/53	20.0	11.0	30.0	14.0	1.68	19	
9/29/53	97.0	20.0	37.0	7.0	9.33	20	**
BLOOD PLASMA CAROTENE (μg./100 ml.)—CALVES							
7/ 3/53	225.0	104.0	227.0	100.0	0.05	21	
8/ 4/53	47.0	24.0	51.0	22.0	0.41	21	
9/ 1/53	23.0	8.0	32.0	14.0	1.80	21	
9/29/53	132.0	34.0	42.0	17.0	8.10	21	**
10/27/53	8.0	7.0	4.0	3.0	1.81	21	
11/24/53	11.0	5.0	14.0	6.0	1.29	21	
12/21/53	0.0	....	0.0	....	.....	....	
1/19/54	0.0	....	0.0	....	.....	....	
2/16/54	0.0	....	0.0	....	.....	....	
3/16/54	0.0	....	0.0	....	.....	....	
4/13/54	0.0	....	0.0	....	.....	....	
5/11/54†	8.0	3.0	25.0	10.0	2.28	20	*
BLOOD PLASMA VITAMIN A (μg./100 ml.)—COWS							
7/ 7/53	19.7	2.3	20.0	3.0	0.25	19	
8/ 4/53	27.0	....	31.0	(analyzed by pooled lots)			
9/ 1/53	32.2	3.7	24.7	5.9	3.39	19	**
9/29/53	31.0	....	26.0	(analyzed by pooled lots)			
BLOOD PLASMA VITAMIN A (μg./100 ml.)—CALVES							
7/ 3/53	13.1	3.7	13.9	3.1	0.73	21	
8/ 4/53	15.0	....	20.0	(analyzed by pooled lots)			
9/ 1/53	20.7	3.2	20.6	5.0	0.05	21	
9/29/53	25.0	....	16.0	(analyzed by pooled lots)			
10/27/53	25.9	9.4	18.3	4.6	2.48	21	*
11/24/53	27.0	7.2	16.0	5.6	4.04	21	**
12/21/53	33.5	4.5	14.1	5.4	9.07	21	**
1/19/53	26.6	4.2	10.9	3.5	9.57	21	**
2/16/53	27.6	2.1	13.9	2.0	4.84	20	**
3/16/53	25.8	2.8	14.2	2.8	9.21	19	**
4/13/53	28.2	3.5	9.6	1.2	16.76	20	**
5/11/53	32.5	5.3	19.5	4.9	5.88	20	**
LIVER VITAMIN A (μg./Gm. tissue)—CALVES							
11/24/53	20.9	8.9	2.2	1.0	7.26	21	**
5/ 5/54	17.3	12.2	1.4	0.5	4.32	20	**

† The rise in plasma carotene in the deficient calves on 5/11/54 followed the accidental feeding of some of them on green grass the day before.

* Means are significantly different at the 5% level; ** means are significantly different at the 1% level.

s.d.—standard deviation.

TABLE 3—Silica Content, Specific Gravity, and pH of Urine Samples from Vitamin A-Supplemented and A-Deficient Calves

Date	Supplemented		Deficient		t-value	No. of animals	Significance
	Mean	s.d.	Mean	s.d.			
			TOTAL SILICA (as p.p.m. SiO_2)				
11/10/53	385.0	151.0	244.0	129.0	2.31	20	*
12/ 8/53	503.0	69.0	490.0	99.0	0.38	20	
2/ 2/54	410.0	150.0	441.0	104.0	0.92	19	
			DISSOLVED SILICA (as p.p.m. SiO_2)				
2/ 2/54	282.0	102.0	399.0	102.0	2.63	19	*
			PRECIPITATED SILICA (as p.p.m. SiO_2)				
2/ 2/54	128.0	87.0	42.0	54.0	2.58	19	*
			SPECIFIC GRAVITY				
11/10/53	1.038	0.005	1.037	0.004	0.67	20	
2/ 2/54	1.024	0.012	1.031	0.004	1.89	19	
4/ 9/54	1.032	0.006	1.029	0.008	1.23	19	
			pH				
11/10/53	8.8	0.2	8.6	0.2	1.63	20	
2/ 2/54	7.6	0.6	8.2	0.2	2.68	19	*
4/ 9/54	8.2	0.2	7.6	0.7	3.91	19	**

* Means are significantly different at the 5 % level; ** means are significantly different at the 1 % level.

the remainder of the experimental period. Thus the period of vitamin A deficiency coincided with the usual period of urolithiasis in range steers. In the supplemented calves, this value remained above 25 μg./100 ml. for the same period.

The first liver biopsy was made on November 24, at which time the mean value for the deficient calves was 2.2 μg. per gram, while for the supplemented calves it was 20.9 μg. per gram. When the second biopsy was taken on May 5, both of these values were slightly lower.

Additional evidence for a vitamin A deficiency among the unsupplemented calves was obtained from the night-blindness test conducted at the conclusion of the experiment. At that time, 4 of the 11 calves were definitely nightblind. On the following day, the eyes of the calves were examined. One calf had a corneal ulcer on one eye, 4 had some corneal opacity on one eye, and 1 had some opacity on both corneas. All other eyes appeared normal. The affected eyes were all in the vitamin A-deficient lot.

The choice of tests to be applied to the urine specimens obtained from these two lots of calves was dictated by the very scant knowledge of the factors involved in calculus formation in this type of animal.

Because calculi from range steers are known to consist largely of silica,[39] this substance was determined in each sample on three different dates. In addition, the silica analysis of the samples collected on February 2 included not only total silica but also a separate determination of the silica content of the clear, centrifuged urine as a means of distinguishing the total silica, the dissolved silica, and the precipitated silica. The results of all the silica analyses are summarized (table 3). There are significant differences at the 5 per cent level between the means of these values for the two lots of calves on two different dates. In November, the supplemented calves had a higher total silica, while in February it was the deficient calves which had a higher urinary silica. Also, in February the amount (and proportion) of "dissolved" silica was significantly greater for the deficient lot, contrary to what might be expected if this lot were excreting a urine of lowered silica-retaining capacity associated with stone formation. The explanation of these facts is not apparent.

Since the nature of the organic matter found in the uroliths of range steer is not known, determinations of this material in the urine samples were not made.

Analyses for phosphates, carbonates, oxalates, magnesium, calcium, and other components of urinary stones were not made because of the relative scarcity of these ions in typical range steer calculi.[39]

Increased concentration of urinary solids has often been mentioned as being a possible contributing factor to stone formation. As a measure of urinary concentration, specific gravity determinations were made (table 3). There was no significant difference between the two lots.

Although there is no reason to believe that differences within the pH range of

TABLE 4—Physical and Colloidal Properties of Urine Samples from Vitamin A-Supplemented and A-Deficient Calves

Date	Supplemented Mean	Supplemented s.d.	Deficient Mean	Deficient s.d.	t value	No. of animals	Significance
			Relative Viscosity (Water = 1)				
As voided 2/ 2/54	1.137	0.071	1.158	0.033	0.88	19	
Diluted to Sp. Gr. 1.010 2/ 2/54	1.061	0.015	1.068	0.029	0.73	19	
			Surface Tension (dynes/cm.)				
11/10/53	51.6	0.9	51.2	1.3	0.84	20	
2/ 2/54	51.4	3.0	50.8	1.8	1.48	19	
4/19/54	48.3	1.8	47.3	1.3	1.49	19	
			Colloidal Gold Protection (Units of Protective Activity/ml.)				
3/ 8/54	3.1	2.6	1.9	1.8	1.21	19	
4/19/54	3.2	3.1	2.3	2.8	0.67	19	
			Crystal Formation in Dried Films (Puntriano Numbers)				
3/ 8/54	5.8	0.6	5.3	0.8	1.68	19	
4/19/54	4.2	1.1	4.4	1.4	0.44	19	
			Specific Conductivity (Millimohs)				
11/10/53	28.2	6.8	30.2	6.8	0.52	20	
2/ 2/54	20.2	8.8	19.3	5.9	0.23	19	
4/19/54	21.6	5.9	15.6	4.1	2.72	19	*
			Volume of Sediment (Arbitrary Units)				
11/10/53	4.5	2.2	3.7	1.6	1.04	20	
			Turbidity (Optical Density Readings)				
2/ 2/54	0.53	0.40	0.41	0.29	0.76	19	
4/19/54	0.49	0.28	0.49	0.36	0.01	19	

* Means are significantly different at the 5 % level.

TABLE 5—Microscopic Examination of Urinary Sediments from Vitamin A-Supplemented and A-Deficient Calves

Date	Supplemented Present	Supplemented Absent	Deficient Present	Deficient Absent	*Chi* square	Significance
			Number of Samples Containing Crystals			
2/ 2/54	5	5	11	0	7.224	**
3/ 3/54	9	1	9	2	0.303	
4/19/54	7	3	10	1	1.487	
			Number of Samples Containing "Matrix" Material			
2/ 2/54	6	4	10	1	2.743	
3/ 8/54	10	0	10	1	0.942	
4/19/54	9	1	9	2	0.303	
			Number of Samples Containing "Agglutinated" Crystals			
2/ 2/54	5	5	10	1	4.298	*
3/ 8/54	8	1	7	4	1.682	
4/19/54	6	1	6	4	1.313	
			Number of Samples Containing "Free" Crystals			
2/ 2/54	5	4	5	6	0.202	
3/ 8/54	8	1	7	4	1.682	
4/19/54	7	0	8	2	1.714	
			Number of Samples Containing Orthorhombic Prisms			
2/ 2/54	6	3	3	8	3.104	
3/ 8/54	6	4	7	4	0.029	
4/19/54	3	7	4	7	0.094	
			Number of Samples Containing Short Hexagonal Prisms			
2/ 2/54	6	4	7	4	0.029	
3/ 8/54	5	5	6	5	0.044	
4/19/54	7	3	9	2	0.405	
			Number of Samples Containing Long Prisms			
2/ 2/54	1	9	7	4	6.392	*
3/ 8/54	5	5	4	7	0.399	

* Means are significantly different at the 5 % level; ** means are significantly different at the 1 % level.

bovine urines would influence the precipitation of silica alone, there is a possibility that if stone formation is a coprecipitation of silicic acid with urinary protein, pH might influence to a considerable extent this reaction between a weak acid and an amphoteric substance. Significant differences in the pH measurements appear on two dates (table 3). In February, the urinary pH from the deficient calves was higher than that of the supplemented ones, while in April the reverse was true. Interpretation of these results, therefore, is obscure.

Silicic acid, being a hydrophobic colloid, must be held in solution either by the electrical charge it carries or by the protection of other colloidal material. The high concentration of electrolytes in urine would suppress the electrostatic stabilization of the silicic acid, so it is probable that stabilization is the result of the presence of protective colloids or other surface-active materials. Not knowing the nature of these substances, some general measurements were made of the physical and chemical properties of the urine which might be related to them. These measurements included viscosity, surface tension, colloidal gold protection, and crystal formation in dried films of urine. The results of these measurements are recorded (table 4). In none of these values was there any significant differences between the two lots of calves.

Electrolytes, in sufficient concentration, promote the precipitation of hydrophobic colloids, even in the presence of protective colloids. The specific electrical conductivity of the urine was used as a measure of the electrolyte concentration. On the last of the three sampling dates, the supplemented lot had a significantly higher conductivity than the deficient lot (table 4). This trend is the reverse of what might be expected in a urine tending to form stones.

Finally the actual tendency of the urine to form precipitates and the nature of the precipitates were investigated. In one collection the volumes of the precipitate were measured, but on subsequent collections a turbidity measure was used instead (table 4).

Microscopic examination of the urinary sediments revealed wide variations in the morphological pattern of the individual specimens (table 5). The principal objects observed were squamous epithelial cells and three types of crystals: orthorhombic prisms, short hexagonal prisms, and long slender prisms. The chemical composition of these crystals is not yet known, but they were found not to be the same as crystals described for human urine.[61] Moreover, they were readily soluble in dilute acid, a property not shared by range steer urinary calculi. In some urine specimens there appeared clumps of finely granular material, which are here termed "matrix." Often the crystals were stuck to this material or embedded in it. When this was so, the crystals were called "agglutinated." When the crystals were not agglutinated, they were termed "free." Some samples contained both agglutinated and free crystals. The *chi*-square test was used for significance. Although significant differences appear three times in the February collection, these differences were not observed on subsequent collections. Therefore, it is unlikely that these differences are related to the vitamin A deficiency, which was as severe at the time of the later collections as it was for the first.

General Discussion

Those who have considered the etiological factors of urolithiasis in cattle have generally concluded that urinary calculi are caused by several interacting factors, one of which may be a vitamin A deficiency. The evidence presented in this paper demonstrates that vitamin A deficiency of itself is not sufficient to cause urolithiasis in cattle. Moreover, it is shown that a vitamin A deficiency is not necessary since a large proportion of calculus cases occurs in animals which from their dietary history should not be deficient in vitamin A and which from blood analysis are proved not to be deficient.

However, proof that a vitamin A deficiency may not sometimes be a contributory factor in urolithiasis will have to wait until the other factors in calculus formation are known. It should then be possible to evaluate the effect of vitamin A deficiency in increasing the incidence of calculosis when the other conditions are right.

The choice of tests to be applied to the urine samples from the vitamin A-deficient and the A-supplemented calves was difficult because of the lack of knowledge of the physical-chemical conditions involved in calculus formation. It is quite possible that a critical test was overlooked. If the

TABLE 6—Weights and Weight Gains of Vitamin A-Supplemented and A-Deficient Calves

Date	Supplemented		Deficient		t-value	Number of animals	Significance
	Mean	s.d.	Mean	s.d.			
			WEIGHTS (LB.)				
12/ 2/53	375	53	336	55	1.65	19	
12/31/53	401	57	356	57	1.80	19	
4/15/54	448	71	407	64	1.38	19	
5/12/54	468	79	417	65	1.48	19	
			WEIGHT GAINS 12/2/53 TO 5/12/54				
	93	37	81	17	0.95	19	

studies now underway at this laboratory and elsewhere disclose additional information about the conditions for calculus formation, it may be necessary to repeat a vitamin A deficiency experiment in which more appropriate measurements are applied to the urine.

There is a hypothesis, shared by the authors, that subclinical urolithiasis is a normal condition in weaned calves on range, and it is only by chance that the stones grow large enough to cause trouble before they are passed with the urine. The study presented herein makes no direct contribution to the testing of this hypothesis, but it does tend to eliminate the conflicting hypothesis that a vitamin A deficiency is a major cause of urolithiasis in range steers.

SUMMARY

1) The literature reveals no clear relationship between urolithiasis and vitamin A deficiency in cattle.

2) Field studies in Montana show that urolithiasis in range cattle appears at least as frequently among animals receiving presumably adequate supplies of vitamin A as it does among animals on low vitamin A rations.

3) It is not clear whether there is a cause and effect relationship in the occasionally observed association of clinical urolithiasis with a low plasma vitamin A level in range cattle.

4) Comparison of the urine from normal and vitamin A-deficient weaned steer calves on a hay ration failed to reveal any consistent differences with respect to silica content, specific gravity, pH, viscosity, surface tension, colloidal gold protection, crystal formation in dried films, electrical conductivity, turbidity, or the nature of the microscopically observed sediments.

In the few instances when statistically significant differences were observed between the urines of the vitamin A deficient and the supplemented steers, the differences were not repeated or were reversed on other sampling dates, so no interpretation of their meaning was undertaken.

5) It is concluded that a vitamin A deficiency is neither a necessary nor by itself a sufficient condition for urolithiasis in range steers, but the possibility of its being a contributory factor is not eliminated.

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Studies on Trichloroethylene-Extracted Feeds. I. Experimental Production of Acute Aplastic Anemia in Young Heifers

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THE OCCURRENCE, in Minnesota, of many cases of fatal aplastic anemia in cattle[1] induced by the feeding of trichloroethylene-extracted soybean oil meal (TCESOM) provided the impetus for extensive studies at the Minnesota Agricultural Experiment Station on the potential toxicity of this meal and some other products extracted with trichloroethylene. This work afforded the opportunity to compare the relative toxicity of different specimens of this meal, fed at different levels to cattle, to study with several other species the biological effects of TCESOM of a known degree of toxicity to cattle, to investigate the metabolism of trichloroethylene in calves, and to develop a bio-assay for the toxic principle of this product. In addition, it became possible to investigate the clinical, hematological, and pathological aspects of TCESOM intoxication and to make biochemical observations on blood, urine, and bonemarrow of experimental animals.

The earlier literature on intoxication in cattle, as a result of feeding this meal, has been reviewed in previous papers.[1-3] In spite of considerable experimental work, the chemical nature of the toxic material in this product, and the mechanism of its biological effect, are completely unknown. No information has been published on conditions required to produce toxic specimens of TCESOM or on the reason why certain specimens of it are relatively nontoxic to cattle.

The present paper summarizes our experience with the feeding of two different types of specimens of TCESOM to young heifers and on the experimental production of acute aplastic anemia. Other phases of the investigations will be presented in subsequent publications.

MATERIALS AND METHODS

Twenty-five healthy heifers weighing 250 to 300 lb. were held for a six-week quarantine and control period and were fed the pretrial ration (table 1). After suitable base-line clinical, hematological, and biochemical data had been collected, the heifers were separated at random into five groups designated A, B, C, D, and E and fed the experimental rations (table 1).

Clinical examinations on all animals were made daily and hematological examinations were made at weekly intervals. Blood was collected from a jugular vein and portions were used immediately for thrombocyte counts, for measurement of coagulation time, prothrombin, and for observations on clot retraction. Smears for differential leukocyte determinations were made from fresh blood. Oxalated blood was used for determination of hematocrit, erythrocyte sedimentation rate, hemoglobin, erythrocyte and total leukocyte counts.

The following procedures were used, among standard methods,[4] for hematological determinations: oxyhemoglobin for hemoglobin, Rees-Ecker's for direct thrombocyte counts, Wright's stain for differential leukocyte counts, Sanford's for erythrocyte fragility, Lee and White's for coagulation time, and Duke's for bleeding time. Clot retraction was measured by observing, in 30-minute intervals, the time required for definite contraction of the clot to occur at 37 C.

Blood was obtained from all animals at weekly intervals for the determination of lactic acid,[5] pyruvic acid,[6] urea nitrogen,[4] plasma ascorbic acid,[7] and serum bilirubin.[8] In addition, periodic determinations were made on the blood from the control group A and from group D, during a period when blood changes characteristic for marked aplastic anemia were present, for the following components: thiamine,[9] serum calcium,[4] inorganic phosphorus,[4] magnesium,[10] nonprotein nitrogen,[4] creatine,[3] creatinine,[3] uric acid,[4] amino nitrogen,[4] total cholesterol,[3] total fatty acids,[3] plasma albumin, plasma globulin, and fibrinogen,[11] using the Hill-Trevarrow method of fractionation.[12]

This series of studies is from the School of Veterinary Medicine and Department of Agricultural Biochemistry, Institute of Agriculture, University of Minnesota, St. Paul. References to all literature cited will be found at the end of paper VII (p. 453).

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TABLE 1—Experimental Rations and Amounts Fed to Heifers

Components	(Pretrial)*	Groups (5 animals each) A	B	C	D	E
	Pounds per day					
Corn and cob meal **	0.75	0.75–1.25	0.75–1.25	0.75–1.25	0.75–1.25	0.75–1.25
Crushed oats **	0.75	0.75–1.25	0.75–1.25	0.75–1.25	0.75–1.25	0.75–1.25
Total soybean oil meal	3.00	3.00	3.00	3.00	3.00	3.00
Type:† Expeller	3.00	.....	.....	.....	.....	.....
Hexane-extracted	.....	3.00	.....	.....	2.00	2.00
TCESOM-1	.....	.....	.....	3.00	1.00	.....
TCESOM-2 and 3	.....	.....	3.00	.....	.....	1.00
Steamed bone meal	0.05	0.05	0.05	0.05	0.05	0.05
Trace-mineralized salt	0.05	0.05	0.05	0.05	0.05	0.05
Good quality alfalfa or alfalfa and brome hay—*ad libitum* to all groups						

* Twenty-five heifers for six weeks; ** the higher level was started on the experimental day 85 in order to maintain growth at the same rate.

† The soybean oil meals were obtained from the following sources: expeller-type, purchased on the open market; hexane-extracted, purchased on the open market; TCESOM-1, trichloroethylene-extracted soybean oil meal (TCESOM) produced in October, 1950, from 1950 crop of soybeans and obtained from remaining stocks from a farm where cattle losses had been associated with the feeding of this meal (this specimen had been produced one year prior to the time it was fed in this study); TCESOM-2, trichloroethylene-extracted soybean oil meal produced in August, 1951, from 1950 crop beans, was produced in the same extraction plant under operating conditions which the manufacturer believed to be identical with those prevailing when TCESOM-1 was produced. This specimen was fed in this experiment for about five months followed, for the remaining period of observation, by TCESOM-3. The latter specimen was made in February, 1952, from 1950 crop beans in a different extraction plant which, however, used the same type of equipment and similar operating conditions as the plant which produced TCESOM-1 and TCESOM-2.

With blood collected aseptically, cultures were made on specimens from animals of group D at weekly intervals during a nine-week period when blood changes characteristic of marked aplastic anemia were present. Ten milliliters of blood was placed into each of two St. Louis bottles containing 50 ml. of Kracke's blood culture medium. One bottle was incubated aerobically at 37 C. for 72 hours under 20 per cent CO_2, the other anaerobically.

Urine was collected at nine weekly intervals from animals in group D, during the time blood changes characteristic of marked aplastic anemia were present, and analyzed by standard procedures[4] for specific gravity, protein, reducing sugars, acetone, bile, and occult blood. The sediment obtained from centrifuged specimens was examined microscopically.

Gross and microscopic examinations for pathological changes were made on 5 animals that died (group C) and on 5 others that were killed during the experiment. One animal from group A was slaughtered on the seventy-third experimental day so that control tissues would be available for histological comparison with tissues from the animals that died in group C. One animal was slaughtered from each of the remaining groups on the 212th day of the experiment. The further progress of the 15 animals remaining on the 214th day of the experiment will be discussed in a subsequent paper.

OBSERVATIONS

Clinical Observations. — The heifers of different breeds responded to the treatments in the same manner. They consumed the entire allotment of concentrate fed each day. During the first four weeks, they remained healthy and made satisfactory weight gains (table 2). No signs of disease were noticed in any of the animals until the twenty-eighth day when 1 animal from group C, fed 3 lb. of TCESOM-1, developed fever, a serous nasal discharge, signs of abdominal distress, and petechial hemorrhages on the visible mucous membranes.

TABLE 2—Summary of Observations on Young Heifers Fed Hexane-Extracted Soybean Oil Meal, TCESOM-1, or TCESOM-2 and 3

Items compared	Groups (5 animals each) A	B	C	D	E
Soybean oil meal type	HEXSOM	TCESOM-2, 3	TCESOM-1	TCESOM-1	TCESOM-2, 3
Daily intake (lb.)	3.0	3.0	3.0	1.0	1.0
Av. wt. start (lb.)	328	320	333	329	337
Av. wt. 34 days (lb.)	383	381	378	384	404
Av. wt. 214 days (lb.)	687	662	.....	684	700
First blood change (days)	None	47	24	30	46–53
Animals with blood changes	None	All	All	All	All
Animals with symptoms	None	4	All	All	3
Died (days)	None	None	All (35–55)	None	None
Destroyed (days)	1(73) 1(212)	1(212)	.....	1(212)	1(212)
Blood picture at end of experiment	Normal	Normal	.....	Aplastic anemia	Normal

HEXSOM, hexane-extracted soybean oil meal; TCESOM, trichloroethylene-extracted soybean oil meal.

TABLE 3—Summary of the Biochemical Data of the Blood of Heifers, Groups A, B, D, E, in the Experimental Production of Acute Aplastic Anemia

	A			B			D			E			Significance[4]
	N[1]	M[2]	SE[3]	N	M	SE	N	M	SE	N	M	SE	
Lactic acid (mg./100 ml.)	112	8.78	0.49	140	9.07	0.44	139	8.2	0.44	135	9.1	0.44	NS
Pyruvic acid (mg./100 ml.)	112	1.01	0.04	138	0.98	0.04	138	1.00	0.04	134	1.03	0.04	NS
Thiamine (μg./100 ml.)	18	4.99	0.28	20	5.94	0.27	20	4.62	0.27	20	4.4	0.27	NS
Ascorbic acid (mg./100 ml.)	97	0.53	0.01	125	0.54	0.01	119	0.52	0.01	114	0.48	0.01	NS
Urea (mg./100 ml.)	87	22.8	0.56	105	23.9	0.51	112	22.1	0.49	96	22.3	0.53	NS
Bilirubin (mg./100 ml.)	82	0.19	0.01	104	0.19	0.01	99	0.16	0.01	90	0.20	0.01	A,B,E > D
Clotting time (min.)	100	18.3	0.89	139	17.1	0.76	144	23.0	0.74	145	17.8	0.74	D > A
Erythrocyte (at start)	96	0.634	0.011	135	0.658	0.009	138	0.644	0.009	128	0.637	0.01	NS
Fragility (% NaCl) (at end)	96	0.470	0.008	135	0.477	0.007	138	0.489	0.007	128	0.473	0.007	NS

[1] Number of determinations; [2] mean of results; [3] standard error of means; [4] NS, no significant difference among groups. A, B, E > D: means of groups A, B, and E significantly higher than mean of group D (probability of chance occurrence less than 5%); D > A: mean of group D higher than mean of Group A (probability of chance occurrence less than 5%).

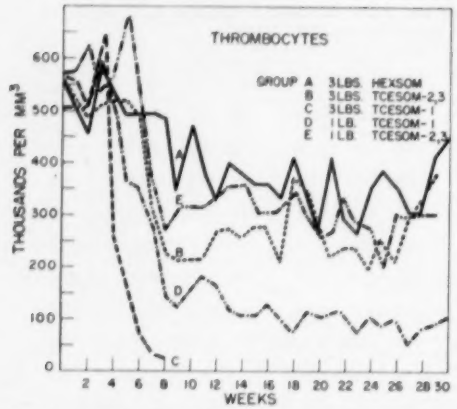
Within the next three days, all of the animals of group C became ill. The following signs were observed in these animals sometime during the course of the disease which varied from seven to 24 days in length: anorexia, tachypnea, tachycardia, pyrexia with temperatures ranging from 103.6 to 108.2 F., serous and sometimes sanguineous nasal discharge, slight to moderate expistaxis, evidence of acute abdominal distress (grinding of the teeth, moaning, staring at their flanks, kicking at their abdomens), petechial and echymotic hemorrhages on the exposed mucous membranes and of the skin over the thin-skinned areas, flecks of blood in the feces, pale and icteric mucous membranes during the latter stages of the disease. All of the animals of group C died between the thirty-fifth and fifty-fifth day of the experiment.

Between the forty-seventh and fifty-sixth experimental day, 4 animals in group B and all of the animals in group D exhibited the following signs of illness: anorexia, tachypnea, tachycardia, pyrexia, serous nasal discharge, petechial hemorrhages on the visible mucous membranes, and evidence of abdominal distress. Most of these animals were noticeably ill for one to three days. One animal in group D was ill for eight days. Three animals in group E had nasal discharges and were slightly depressed for several days. They did not develop the marked symptoms of illness encountered in the other groups, however, and all recovered quickly with no appar-

TABLE 4—Summary of the Biochemical Data of the Blood of Heifers, Groups A and D, in the Experimental Production of Acute Aplastic Anemia

	A			D			Significance of difference[4]
	N[1]	M[2]	SE[3]	N	M	SE	
Nonprotein-N (mg./100 ml.)	23	35.7	0.63	29	34.6	0.56	NS
Uric acid (mg./100 ml.)	16	3.14	0.17	19	2.73	0.15	NS
Creatine (mg./100 ml.)	20	1.03	0.08	24	1.07	0.07	NS
Creatinine (mg./100 ml.)	20	2.55	0.21	24	2.37	0.19	NS
Amino-N (mg./100 ml.)	16	5.64	0.10	19	5.73	0.09	NS
Calcium (mg./100 ml.)	23	10.2	0.23	29	10.1	0.21	NS
Phosphorus (mg./100 ml.)	23	7.3	0.23	29	7.5	0.20	NS
Magnesium (mg./100 ml.)	23	1.8	0.11	29	1.9	0.10	NS
Cholesterol (mg./100 ml.)	23	174.2	5.9	29	197.2	5.3	P = <5%
Fatty acids (mg./100 ml.)	23	248.2	3.1	29	256.2	2.7	NS
Albumin (Gm./100 ml.)	23	3.3	0.11	29	3.0	0.10	NS
Globulin (Gm./100 ml.)	22	3.0	0.15	29	3.6	0.13	P = <5%
Fibrinogen (Gm./100 ml.)	22	0.55	0.03	29	0.49	0.02	NS
Plasma proteins (Gm./100 ml.)	22	7.1	0.10	29	7.1	0.08	NS
Albumin-globulin ratio	22	1.13	0.06	29	0.88	0.05	P = <5%

[1] Number of determinations; [2] mean of results; [3] standard error of mean; [4] NS, no significant difference; P = <5%, probability that difference is due to chance is less than 5 per cent.

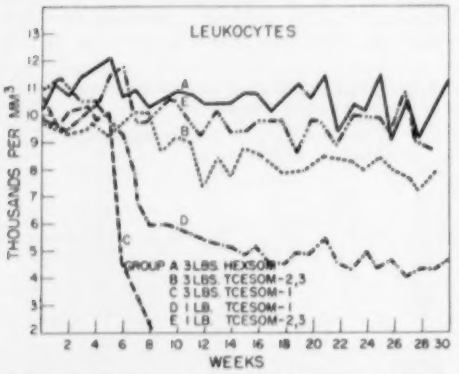


Graph 1—Changes of thrombocyte counts of heifers fed different specimens of trichloroethylene-extracted soybean oil meal at different levels. The control group A was fed hexane-extracted soybean oil meal.

ent permanent ill effects. None of the signs of disease referred to above was ever encountered in the animals of group A.

In all animals of group D, the signs of intoxication referred to above recurred periodically, followed in each case by apparently complete clinical recovery. The animals of groups B and E, in contrast, had no recurrence of the signs of illness and they appeared normal at the end of the experiment. The weight gains of the four surviving groups were essentially the same (table 2).

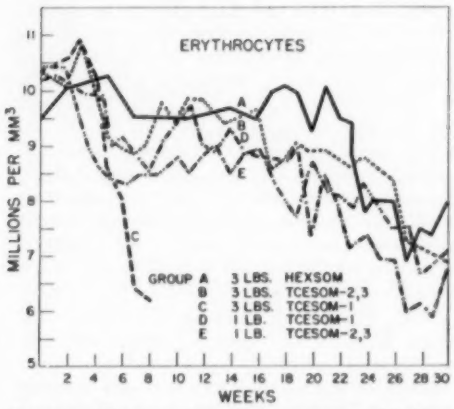
Bacteriological Observations.—Results of the bacteriological examinations of the blood obtained from the animals of group D at nine weekly intervals, during a period when blood changes characteristic of aplastic anemia were present, revealed occasional presence of various types of bacteria; but, no correlation could be made between these isolations and the occurrence of clinical signs of disease such as fever, anorexia, and nasal discharges. It is probable that invasion by microorganisms, which occurs in fatal cases of TCESOM intoxication, is a terminal phenomenon and that it does not cause the common clinical signs of the disease.



Graph 2—Changes of leukocyte counts of heifers fed different specimens of trichloroethylene-extracted soybean oil meal at different levels. The control group A was fed hexane-extracted soybean oil meal

Hematological Observations.— The first blood change noticed on the twenty-fourth day was a marked reduction in the thrombocytes in all of the animals in group C. Thrombocytopenia was followed by a rapidly progressing reduction in all of the cellular elements of the blood which continued until the animals died in 35 to 55 days. Leukopenia and erythropenia occurred within several days after the appearance of thrombocytopenia (graphs 1 to 3). The reduction in neutrophils was proportionately greater than the reduction in the number of lymphocytes; this produced a marked relative lymphocytosis. No increase in the numbers of immature erythrocytes was detected in any of the animals. Little change occurred in the mean corpuscular hemoglobin; the mean corpuscular volume tended to be slightly increased during the later stages of the disease. No marked alterations were found in the erythrocyte fragility or in the prothrombin content of the blood. The erythrocyte sedimentation rate was slightly increased. Four of the 5 animals in group C developed a markedly prolonged blood-clotting time shortly before death. Bleeding times were prolonged in all animals after marked blood changes had occurred. In several instances, the bleeding times exceeded 24 hours. Clot retraction was delayed after the appearance of thrombocytopenia and retraction did not occur after the thrombocyte counts were greatly reduced.

Blood changes which were characteristic of aplastic anemia developed after 30 days in all of the animals in group D fed 1 lb. of TCESOM-1 per day. There was a great difference, however, between group C and group D in the rate at which the changes occurred. In group D, they were slowly progressive in contrast to the rapidity with



Graph 3—Changes of erythrocyte counts of heifers fed different specimens of trichloroethylene-extracted soybean oil meal. The control group A was fed hexane-extracted soybean oil meal.

which they took place in group C. No marked increase in blood clotting time occurred in group D. However, the bleeding times were always prolonged after marked thrombocytopenia developed. Retardation of clot retraction and failure of the clot to retract *in vitro* could be closely correlated with the severity of the thrombocytopenia.

All of the animals in groups B and E, fed 3 lb. and 1 lb. of TCESOM-2, respectively, developed thrombocytopenia between the forty-sixth and the fifty-third days. Shortly thereafter, slight leukopenia and slight anemia developed in both groups. These changes were considerably more marked in group B than in group E. No other hematological change was noticed in these groups.

None of the animals in group A, fed 3 lb. of hexane-extracted soybean oil meal, developed similar blood changes except for a decrease in the erythrocyte count to levels which are within the normal range observed for animals of this age.

Biochemical Observations.—Urine analyses of specimens collected from group D consistently gave positive tests for bile and for occult blood. All other analyses gave results which were in the normal range for heifers of this age and there was no difference between the various groups.

The results of blood analyses (tables 3 and 4) revealed no significant differences in any of the determinations except that the values for cholesterol, globulin, and clotting time were significantly greater for group D than for the other groups at the 5 per cent level of probability. The albumin-globulin ratio, and bilirubin values for group D were significantly less at the same level of probability.

Necropsy Observations.—Gross and microscopic observations revealed pathological changes identical to those which occurred in field cases of aplastic anemia resulting from consumption of TCESOM.[12]

Discussion

The production of fatal, acute aplastic anemia in cattle under closely controlled experimental conditions confirmed that, as on farms,[1] this disease was induced by TCESOM. It also established that (1) the severity of the disease is related to the amount fed, (2) not all specimens of this feed have the same degree of toxicity, (3) young cattle may appear to be clinically normal and make good weight gains in spite of the consumption of considerable quantities of toxic specimens, and (4) detailed hematological studies provide the best criteria for diagnosis of intoxication caused by this feed, particularly when the intake of toxic soybean oil meal or the degree of its toxicity are of relatively low order.

Summary and Conclusions

1) Acute aplastic anemia has been produced in young heifers by feeding trichloroethylene-extracted soybean oil meal (TCESOM).

2) The principal clinical and hematological features of the disease and the necropsy findings were essentially the same as those observed on farms where this meal was fed to cattle.

3) There were marked differences in the degree of toxicity produced by two different specimens of this meal.

II. The Effect of Feeding Different Levels of Trichloroethylene-Extracted Soybean Oil Meal to Young Heifers—Experimental Production of Chronic Aplastic Anemia

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AN INSIDIOUS, chronic form of an aplastic anemia in cattle associated with the consumption of trichloroethylene-extracted soybean oil meal (TCESOM) was observed during a period when many cases of acute TCESOM intoxication occurred in Minnesota.[1] The majority of the affected animals did not exhibit clinical signs of disease until after prolonged consumption or until one to eight months after the cessation of the feeding of this meal. In contrast to the animals with acute cases, which developed a striking hemorrhagic syndrome,[1, 12] the chief clinical signs of the chronic intoxication were the eventual development of progressive weakness and anemia, characterized by a normochromic-normocytic anemia, a leukopenia, but often a normal thrombocyte count.[1] The animals with the acute cases presented a normochromic-normocytic anemia, leukopenia, and thrombocytopenia, hence a syndrome of pancytopenia.

During our initial experiments on intoxication of young heifers,[14] it became apparent that the clinical and hematological aspects of the disease were profoundly affected by the quantity as well as the type of TCESOM fed. It became possible, therefore, to attempt the experimental production of various degrees of intoxication, ranging from the acute, fatal syndrome to the chronic, nonfatal case. Experimental production of a chronic, slowly progressing state of aplastic anemia induced by this meal was desired for several reasons. First, it was clearly desirable to determine if complicating factors may have affected the clinical course or development of the many cases observed on farms. Secondly, and of much greater potential importance, there was the possibility that the induction period of incipient aplastic anemia might be spread over a long period, which would afford opportunity for intensive study of animals during a time when they were clearly under the influence of the toxic agent but did not show outward signs of intoxication. Thirdly, the results of such observations might be of particular importance for the recognition of the effects of feeding trichloroethylene-extracted products in which the level of toxicity is too low to be readily recognizable by short feeding tests but which, nevertheless, might be potentially dangerous for cattle and perhaps for other species. Finally, in the absence of knowledge concerning the mechanism by which TCESOM precipitates the metabolic disturbance which leads eventually to the production of aplastic anemia, an investigation of various biochemical and physiological factors in chronically intoxicated cattle might disclose criteria which could be applied to the early diagnosis of aplastic anemia in this and other species.

This paper summarizes the attempts to produce different degrees of intoxication in young heifers by: (1) the continuous feeding of different levels of a highly toxic specimen of TCESOM; (2) the continuous feeding at two levels of two different specimens of a relatively low order of toxicity; (3) intermittent feeding of intermediate levels of a highly toxic specimen.

MATERIALS AND METHODS

Soybean Oil Meal and Rations.—During the preceding study,[14] and this one, several different specimens of TCESOM, produced in commercial plants in Minnesota, were used. Important features concerning their history are summarized (table 1). Hexane-extracted soybean oil meal (HEXSOM) and expeller-type soybean oil meal (EXSOM) were purchased on the open market. Except as indicated in the text, the rations (table 2) were fed to these animals continuously until they died or were slaughtered for necropsy.

Animals.—This study involved in part a continuation of the observations made on animals pre-

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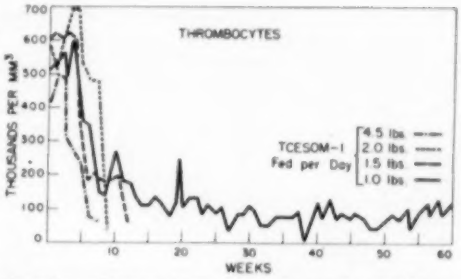
The biochemical analyses were performed by N. S. Mizuno and V. Perman assisted with the handling and management of the animals. Their competent and devoted assistance is gratefully acknowledged.

viously used. There were available 15 normal-appearing, 700-lb. heifers (groups A, B, D, and E from previous study) which had continuously consumed soybean oil meal for 213 days.[18] Two days before the start of the work described in this paper, 1 representative heifer each from groups A, B, D, and E had been slaughtered and examined for pathological changes. The animals in groups A, B, and E had blood counts which were essentially normal. Those in group D showed low values for thrombocytes, erythrocytes, and hemoglobin (about 70% of normal), leukopenia (about 40% of normal), and a relative lymphocytosis, but normal clotting time and a normal prothrombin content similar to that found in incipient aplastic anemia. All animals of group D had shown intermittent periods of anorexia, depression, slight nasal and rectal hemorrhage, and petechial hemorrhages in the oral, conjunctival, and vaginal mucous membranes prior to the start of the current observations.

The other 15 animals, in groups F, G, H, K, and M (tables 2, 3, and 4), were purchased on the open market. After three to four weeks' observation, they were started on the rations shown in table 2. Some of these animals served as controls for those used in the study on intermittent feeding of TCESOM.

To observe the effects of intermittent feeding of this meal, 3 animals were utilized simultaneously with those of groups G and H. The types of soybean oil meal fed and the feeding schedule are shown (table 5).

Tests and Analyses.—The following hematological examinations were made at weekly intervals in all animals of groups D and G and every two weeks in animals in groups A, B, and E: hematocrit, hemoglobin, erythrocytes, leukocytes, differential leukocytes, thrombocytes, erythrocyte sedimentation rate, and clot retraction time. Determinations of the prothrombin content were made periodically with blood from animals in groups A and D. Erythrocyte fragility was determined every two weeks for groups A and D. In addition,



Graph 1—Changes in thrombocyte counts of heifers fed 4.5, 2.0, 1.5, 1.0 lb. of trichloroethylene-extracted soybean oil meal (TCESOM-1) per day.

coagulation times were determined at weekly intervals for group G. From the beginning of the experiment until the animals in group D had died, the following analyses were performed periodically on the blood of the animals of groups A, B, and D: lactic acid, pyruvic acid, uren nitrogen, nonprotein nitrogen, creatine, creatinine, uric acid, amino nitrogen, cholesterol, total fatty acids, serum calcium, plasma inorganic phosphorus, serum magnesium, plasma bilirubin, plasma ascorbic acid, albumin, globulin, and fibrinogen. The blood of the animals of group D was cultured at weekly intervals. The methods for collection of blood and the analytical procedures were those used in the preceding study.[14] In addition, periodic analyses were made of 12-hour urine specimens collected from groups A and D for organic and inorganic sulfates[16] and glucuronosides.[15] A few urine specimens were subjected to chromatographic survey of the 2,4-dinitrophenylhydrazine derivatives of carbonyl compounds.[17] Determinations of blood pantothenic acid were made periodically on animals of groups A and D[18]

Periodic tests for abnormalities of liver function[4] by determination of galactose tolerance,

TABLE 1—Specimens of Trichloroethylene-Extracted Soybean Oil Meal Used to Produce Chronic Aplastic Anemia in Heifers

Specimen*	Time when processed	Soybean harvest	Approximate time between harvesting and processing (months)	TCESOM plant	Produced acute aplastic anemia
TCESOM-1	Oct. 1950	1950	0–1	A	Yes
TCESOM-2	Aug. 1951	1950	10–11	A	No
TCESOM-3	Feb. 1952	1950	16	B	No
TCESOM-4	Dec. 1952	1951	14	B	No
TCESOM-5	Dec. 1952	1952	2–3	B	Yes

* Specimens 1 and 2 were produced in the same plant under operating conditions which were similar, if not identical, as far as can be ascertained. Specimen 1 was procured from remaining stocks of TCESOM on a farm where several cases of acute aplastic anemia were observed in the spring of 1951 among cattle fed this meal. It was also used in the studies previously reported.[18] Specimens 3, 4, and 5 were produced in another extraction plant. Plants A and B used the same type of equipment.[20]

Specimens 4 and 5 were produced in the same plant on the same day under presumably identical operating conditions. In other studies, specimen 4 was fed to two 100-lb. calves for 96 days. They consumed, during this time, 137 and 159 lb., respectively, of this specimen and remained normal, including hematological criteria. Specimen 5 was fed to two similar 100-lb. calves: these animals produced the typical syndrome of aplastic anemia and died in 38 and 45 days after consuming 28 and 42 lb. of specimen 5, respectively.

TCESOM—trichloroethylene-extracted soybean oil meal.

TABLE 2—Rations (lb.) Fed to Young Heifers per Day*

Group	Animals: No. per treatment	Corn and cob meal	Crushed oats	TCESOM specimen: 1,	TCESOM specimen: 2, 3, 4	TCESOM specimen: 5	HEXSOM
M	3	1.5	1.5	4.5	....	....	....
K	3	1.5	1.5	....	....	4.5	....
B	4	1.25	1.25	....	3.0	....	....
A	3	1.25	1.25	....	....	....	3.0
F	1	1.5	1.5	....	....	....	2.0
F	1	3.0	....	2.0	....	....	....
F	1	1.5	1.5	2.0	....	....	....
F	1	....	3.0	2.0	....	....	....
G and H	3	1.5	1.5	1.5	....	....	....
G and H	2	1.5	1.5	1.5	..	....	1.5
D	4	1.25	1.25	1.0	....	....	2.0
E	4	1.25	1.25	....	1.0	....	2.0

* In addition to the components shown in the table, all animals received lequate daily supplements of steamed bonemeal and trace-mineralized sodium chloride mixed with the concentrate and good quality alfalfa or alfalfa-brome hay *ad libitum*.

cephalin-cholesterol flocculation, thymol turbidity, urinary urobilinogen, bromsulphalein excretion, and rose bengal excretion were made on the animals in group G. The galactose tolerance test was performed by measuring the reducing sugars in the blood 20, 40, 80, and 120 minutes after the intravenous administration of a 50 per cent solution of D-galactose at a rate of 1 ml. per kilogram of body weight during a five-minute period. The rose bengal test was made by measuring the concentration of this compound in the plasma two and six minutes, respectively, after the intravenous administration of 20 mg. of rose bengal. The bromsulfalein test was made by measuring the concentration of this dye in the blood serum 10, 20, 40, and 60 minutes following slow intravenous injection at a rate of 2 mg. per kilogram of body weight.

Adrenal and pituitary-adrenal function tests according to Thorn [19] were made periodically in animals of groups A, D, and G. For this purpose, epinephrine hydrochloride was injected intravenously at the rate of 4 ml. of a 1:1000 solution into some animals and 200 mg. of adrenocorticotrophic hormone (Armour standard) were administered intramuscularly to others; two and one half, four, and six hours after these injections, determinations were made of the eosinophils, plasma ascorbic acid, serum cholesterol, and blood glucose. Glucose tolerance was measured by determination of blood-reducing sugar 15 minutes, one, two and one half, four, and six hours after the intravenous administration of 150 ml. of a 40 per cent solution of D-glucose. As a possible indication of the effect of these large doses of glucose on the pituitary-adrenal system, changes in the plasma ascorbic acid, the blood serum cholesterol, and the eosinophils were determined.

Biopsy specimens of bone marrow were removed at weekly intervals from the second or third sternebra of the animals in group D with a trocar and a 10-ml. dry syringe. Stained smears from these specimens were examined microscopically.

All animals which died or were slaughtered were examined for gross and microscopic pathological changes.

RESULTS AND DISCUSSION

Continuous Feeding of a Highly Toxic Specimen of TCESOM.—Every animal which consumed TCESOM–1 at a level of 1 lb. or more per day developed typical symptoms of aplastic anemia. The results of this and the preceding experiment [14] (table 3) show that 17 of 18 animals which consumed this meal at a daily level of 1 lb. or more died or became moribund. The only exception was animal 1097 which, after the first signs of blood dyscrasia became apparent, frequently refused to eat most or all of this meal. When this animal was slaughtered 51 days after the start of the experiment, her blood picture was characteristic of incipient aplastic anemia and some hemorrhages were observed at necropsy. The data also show (table 3) that, when 2 lb. or more of this product was fed, death occurred in from four to eight weeks regardless of the total amount of meal consumed. However, when the daily intake was only 1½ lb. per day, death did not occur until after 12 to 13 weeks, and with the lower level of 1 lb. per day, the heifers survived for ten to 19 months. Within the range of levels of TCESOM–1 tested, signs of blood dyscrasia, that is, thrombocytopenia and leukopenia, became apparent in three to four weeks. The animals consuming the largest daily amount of TCESOM developed signs of blood dyscrasia most rapidly (table 3). However, because the blood counts were not made daily during this period, the quantitative significance of such a relationship should not be stressed.

All of the animals fed TCESOM–1 continuously until death developed thrombocytopenia, leukopenia, neutropenia, and a relative lymphocytosis. The intoxication

was prolonged in animals fed 1.0 lb. per day and these also developed anemia. The changes which occurred in the thrombocyte counts of animals fed 4.5, 2.0, 1.5, and 1.0 lb. of this meal continuously until they died are shown in graph 1. In animals fed 4.5 lb. there occurred, shortly after the twentieth day of feeding, a rapid and progressive decrease in thrombocytes. Animals fed 2.0 lb. per day developed thrombocytopenia after 26 and 27 days, while those fed 1.5 and 1.0 lb. per day developed thrombocytopenia in about four weeks.

Continuous Feeding of TCESOM of Relatively Low Toxicity.—The results obtained with the animals of group B fed daily in succession 3 lb. per day of TCESOM–2, TCESOM–3, and TCESOM–4 and with group E fed in succession 1 lb. per day of TCESOM–2 and TCESOM–3, respectively, are shown (table 4). The animals of both of these groups showed the first signs of blood dyscrasia seven to eight weeks after the start of the initial experiment.[14] In the animals of group E, the blood picture subsequently became normal again and maintained itself in this condition until the time the animals were slaughtered, about 12 months after the start of the experiment. One animal in this group died after ten months from causes not related to the feeding of this meal. The intake of toxic material from TCESOM–2 or 3, when fed at a level of 1 lb. per day, was evidently not sufficient to bring about a permanent depression of the formation of leukocytes and thrombocytes. However, when these same two specimens, as well as TCESOM–4, were fed at a level of 3 lb. per day, a chronic leukopenia and thrombocytopenia became established in 3 of 4 animals. This condition persisted until they were slaughtered about 27 months after the start of the feeding. Three of 4 animals in this group were bred and produced calves. Pregnancy apparently did not aggravate the blood dyscrasia, and the blood counts in the calves were normal at birth and in the subsequent suckling period. In contrast to some observations made on farms, there was no evidence that the calves had been adversely affected by placental transmission of the toxic factor in this meal. This is presumably due to the fact that the level of toxicity in the specimens fed was relatively low.

Feeding of 4½ lb. per day of TCESOM–5 to 500-lb. heifers caused the usual rapid decrease in thrombocytes about the twenty-fifth day of the trial. One of these animals became moribund and was destroyed the eighty-fifth day of the experiment (table 4) at which time it showed signs and lesions of acute aplastic anemia, with severe hemorrhage from the body openings. The other 2 animals, however, were more resistant to the toxic effects of this specimen and did not develop clinical signs of acute aplastic anemia by the time they were

TABLE 3 — Relation Between Intake of Trichloroethylene-Extracted Soybean Oil Meal (TCESOM-1) and Criteria of Toxicity

Group	Animal (No.)	Weight at start (lb.)	Daily intake (lb.)	Total intake to occurrence of			
				First blood dyscrasia		Death	
				(days)	(lb.)	(days)	(lb.)
M	1095	271	4.5	21	94	27	121
M	1096	342	4.5	21	94	29 [1]	130 [1]
M	1097	380	4.5	23	103	51 [2]	† [2]
C [3]	898	288	3.0	24	72	35	105
C	901	315	3.0	24	72	36	108
C	900	342	3.0	24	72	40	120
C	899	352	3.0	24	72	51	153
C	902	372	3.0	24	72	55	165
F	947	366	2.0	26	52	33	66
F	944	298	2.0	27	54	33	66
F	945	330	2.0	28	56	36	72
H	1012 [4]	314	1.5	31	46	92	138
H	1014 [4]	339	1.5	31	46	83	124
G	969	388	1.5	31	46	93	145
D [5]	907	300	1.0	30	30	302	302
D	906	354	1.0	30	30	312	312
D	905	315	1.0	30	30	362	362
D	904	334	1.0	30	30	563	563

[1] Slaughtered when moribund; acute aplastic anemia; [2] slaughtered before signs of disease occurred; intake uncertain because of refusal of feed after 23 days; blood picture of acute aplastic anemia; [3] animals of group C from experiment previously reported [14]; [4] fed TCESOM–4 for 19 days (day 15–34); [5] weight at start of experiment and first blood changes in group D refer to observations made in the preceding experiment.[14]

TABLE 4—Effect of Continuous Feeding of Different Specimens of Soybean Oil Meal to to Young Heifers

Animal (No.)	Group	Soybean [1] oil meal	Months fed	Wt. at start (lb.)	Daily Intake (lb.)	First blood change (days)	Termination			
							Cause	After days	Blood picture	Clinical status
1041	K	TCESOM–5	6	495	4.5	27	Slaugh.	181	Leukopenia and thrombocytopenia	Norm.
1042	K	TCESOM–5	3	535	4.5	24	Slaugh.	85	Characteristics of aplastic anemia	Norm.
1045	K	TCESOM–5	6	550	4.5	27	Slaugh.	181	Thrombocytopenia	Norm.
893	B [3]	TCESOM–2	5	354	3.0	47	Slaugh.	786	Leukopenia and thrombocytopenia	Norm.
895		TCESOM–3	10	339	3.0	47	Slaugh.	798	Leukopenia and thrombocytopenia	Norm.
896		TCESOM–4	12	270	3.0	47	Slaugh.	798	Leukopenia and thrombocytopenia	Norm.
897				310	3.0	47	Slaugh.	798	Normal	Norm.
909	E [3]	TCESOM–2	5	385	1.0	53	Slaugh.	371	Normal	Norm.
910		TCESOM–3	8	305	1.0	46	Died [2]	315	Normal	Norm.
911				300	1.0	53	Slaugh.	371	Normal	Norm.
912				327	1.0	46	Slaugh.	371	Normal	Norm.
888	A [3]	HEXSOM		329	3.0	None	Slaugh.	786	Normal	Norm.
889				307	3.0	None	Slaugh.	786	Normal	Norm.
892				339	3.0	None	Slaugh.	786	Normal	Norm.
946	F	HEXSOM		247	2.0	None	Slaugh.	50	Normal	Norm.
967	G	HEXSOM		407	1.5	None	Slaugh.	410	Normal	Norm.
1013	H	HEXSOM		300	1.5	None	Slaugh.	319	Normal	Norm.

[1] *See* table 1 for history of these meals; [2] died from causes apparently not related to feeding; [3] starting weight and first blood changes refer to observations made in the preceding experiment.[24]

TCESOM—trichloroethylene-extracted soybean oil meal; HEXSOM—hexane-extracted soybean oil meal.

slaughtered six months after the start of the experiment.

Table 4 also includes data on the various control animals fed hexane-extracted soybean oil meal for periods comparable to those which the experimental animals were fed TCESOM. The control animals remained normal. While all 3 animals of group A were bred, only 1 conceived; she produced a normal, vigorous calf.

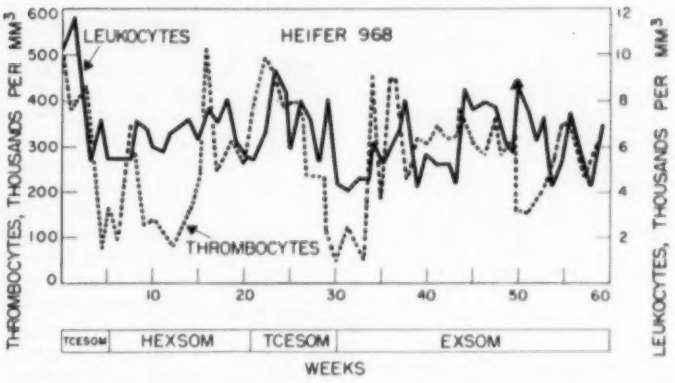
The Effect of Intermittent Feeding of TCESOM.—In an attempt to simulate conditions observed on farms where prolonged blood dyscrasia, unthriftiness, and sudden death from aplastic anemia occurred many months after the feeding of TCESOM–1 had been discontinued, 3 young heifers were fed this meal intermittently (table 5). Animals 966 and 968 consumed 150 lb. during two periods of 40 and 60 days which were interrupted by a 108-day period of HEXSOM feeding. Animal 1015 consumed 138 lb. of TCESOM in 91 days. All of these animals developed thrombocytopenia, leukopenia, and a relative lymphocytosis during the periods when they were fed TCESOM. Marked improvement of the blood dyscrasia followed the change from TCESOM to HEXSOM or EXSOM in all cases. In all of these animals, however, leukocyte counts tended to be lower than before the feeding of TCESOM began. A temporary period of thrombocytopenia also occurred in animal 1015, 20 weeks after cessation of this feeding. The thrombocyte and leukocyte counts of 968 and 1015 are shown (graphs 2 and 3).

These animals remained clinically normal throughout the study and no gross or microscopic lesions were found at necropsy.

TABLE 5—Schedule of Intermittent Feeding of Trichloroethylene-Extracted Soybean Oil Meal to Young Heifers

Period	Soybean oil meal fed per day			Animal and group		
	(type)	(toxicity)	(lb.)	966 G (days)	968 G (days)	1015 H (days)
1	TCESOM–1	High	1.5	40	40	15
2	HEXSOM	None	1.5	108	108	0
3	TCESOM–4	Low	1.5	0	0	19
4	TCESOM–1	High	1.5	60	60	57
5	EXSOM	None	1.5	204	204	228

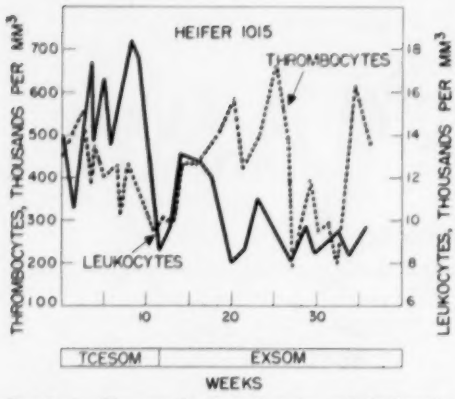
HEXSOM—hexane-extracted soybean oil meal; EXSOM—expeller-type soybean oil meal; TCESOM—trichloroethylene-extracted soybean oil meal.



Graph 2—Changes in thrombocyte and leukocyte counts of a heifer fed trichloroethylene-extracted soybean oil meal intermittently. The periods in which the ration included this meal, hexane-extracted soybean oil meal, or expeller-type soybean oil meal are shown in the bar below the graph.

Degree of Toxicity of Various Specimens of TCESOM.—The results of this study taken in conjunction with the data previously reported [14] have clearly established that there are specimens of TCESOM of relatively high toxicity which, when fed at a level of 2 lb. per day or more, caused, in 35 to 55 days, fatal aplastic anemia in calves weighing initially about 300 lb. On the other hand, there are specimens of a low degree of toxicity, which when likewise fed to such calves did not produce noticeable clinical signs of toxicity for a maximum observation of 798 days involving a total consumption of about 2,400 lb. These latter specimens, however, are not without some degree of toxicity which can be detected by suitable hematological examinations. Thus, in all 5 animals fed 3 lb. per day of one such specimen (TCESOM–2), a temporary blood dyscrasia could be detected after six to seven weeks and the subsequent feeding of two similar specimens (TCESOM–3 and TCESOM–4) produced in 3 of 4 of the same animals a gradual onset of pronounced and persistent thrombocytopenia and leukopenia. Even the feeding of as little as 1 lb. per day of such a specimen of low toxicity (TCESOM–2) produced in all 5 animals an initial temporary blood dyscrasia without, however, causing a later reappearance of this condition during a maximum observation of 371 days (3 animals). It is to be expected that specimens of intermediate, or even higher, or of lower degrees of toxicity have been or could be produced under appropriate conditions. The available evidence presented above, together with other unpublished observations made in these laboratories, indicates that the five available specimens of this meal could be classified into three groups of the following descending order of toxicity: Group 1: high order of toxicity (specimen TCESOM–1); group 2: lower order of toxicity than group 1 (specimen TCESOM–5); group 3: much lower order of toxicity than group 1 or 2 (specimens TCESOM–2, TCESOM–3, and TCESOM–4). No evidence is available which would permit differentiation of the order of toxicity of the three specimens in group 3.

Such a classification prompts an inquiry into the possible causes of the different degrees of toxicity of the various specimens used in this study. Several variables, such as agronomic conditions during growth, variety of soybeans, moisture content at processing, small differences in processing conditions, impurities or decomposition products of the solvent, and others, could not be controlled or appraised for this study. However, the evidence summarized in this paper points to the association of one variable with the degree of toxicity of the soybean oil meal which can not be ignored. This variable is the time interval between harvest and processing of the soybeans as related to the toxicity of the specimens produced. From the results of the preceding study,[14] it became evident that there was apparently a marked difference in the degree of toxicity of specimens TCESOM–1 and 2. Both meals had been produced in the same processing plant under similar, if not identical, conditions, as far as can be ascertained. The outstanding variable between the two specimens was the fact that TCESOM–1 had been produced not later than about one month after harvest of the soybeans



Graph 3—Changes in thrombocyte and leukocyte counts of a heifer fed trichloroethylene-extracted soybean oil meal for 13 weeks followed by 27 weeks of feeding expeller-type soybean oil meal.

whereas TCESOM–2 had been produced from beans of the same crop year but ten to 11 months after harvest. When the supplies of TCESOM–2 neared exhaustion, extraction plant A had suspended operations. However, arrangements were made for the processing of the 1950 crop beans in extraction plant B in February, 1952, at least 16 months after harvest. This specimen (TCESOM–3) was fed at levels of 1 and 3 lb. per day for about eight and ten months, respectively, to heifers that had previously consumed specimen TCESOM–2. No evidence of marked toxicity was obtained during this period. This observation pointed again to the possibility that TCESOM of low toxicity can be prepared by processing soybeans a year or more after harvest. To accumulate further evidence on this point, arrangements were again made with processing plant B in December, 1952, to have 1951 crop soybeans processed to yield specimen TCESOM–4. This was accomplished on the same day, in the same equipment, and with the same solvent used for processing 1952 crop soybeans which yielded specimen TCESOM–5. The data presented (tables 1 and 4) show that the specimen processed about two months after harvesting had a relatively high toxicity while that processed about 14 months after harvesting, from 1951 crop beans, had a low toxicity. These observations, therefore, point to a definite association between a high degree of toxicity of TCESOM and a short interval between harvesting and processing soybeans. Such an association would explain why it was possible, in this instance at least, to obtain specimens TCESOM–3 and TCESOM–4 with the desired low degree of toxicity and specimen TCESOM–5 with the desired relatively high toxicity. It might also explain why earlier trials conducted in 1936–1937 [20] at Cornell University failed to reveal clinical signs of toxicity in cattle fed these specimens. Subsequently, a specimen with a very high degree of toxicity was obtained from processing plant C which produced it in the fall of 1953 from soybeans harvested shortly before.

Hematological Observations.—Of the many determinations performed, only the counts illustrated (graph 1 to 3) and the differential leukocyte counts gave information which was useful in evaluating the effects of the various treatments.

Biochemical Observations.—Of the many different analyses performed on blood and urine of animals fed TCESOM as previously outlined, none gave results which fell markedly outside the range of values obtained from similar analyses made on blood and urine of control animals fed HEXSOM.

Bacteriological Observations.—The blood cultures which were madee periodically, particularly with specimens from animals of group D fed 1 lb. of TCESOM–1 per day, were negative except in a few instances during the terminal stages of intoxication. The positive cultures obtained may reflect bacterial invasion from lesions in the gastrointestinal tract before death. Contrary to the views held by earlier workers, as discussed by Robin,[21] there is no convincing evidence that the syndrome of TCESOM intoxication can be ascribed to a bacteremia.

Functional Tests.—Tests for liver function, adrenal, and pituitary-adrenal function failed to reveal marked deviations from the normal pattern in the animals fed this meal.

Bone Marrow.—Studies of the bone marrow were carried out on numerous specimens removed by biopsy and at necropsy. Associated with the peripheral blood changes of aplastic anemia, there was progressive hypoplasia of the bone marrow involving all hematopoietic elements. Coincident with the first evidence of thrombocytopenia in the peripheral blood, there

were found, in the bone marrow smears, basket cells which were believed to be remnants of megakaryocytes. The number of megakaryocytes observed in the smears diminished after these early changes and few, if any, could be found after marked thrombocytopenia was present. In cases of marked leukopenia, the numbers of myeloid elements in the bone marrow were reduced, with the greatest reduction being in the metamyelocytes. A reduction in the lymphoid elements accompanied the development of absolute lymphocytopenia. Although general hypoplasia was observed, the greatest reduction percentage-wise, as determined by differential counts on bone marrow smears, occurred in the neutophilic and eosinophilic myelocytes as compared to the erythroid and lymphoid elements. In some instances, biopsy specimens appeared to give evidence of accelerated maturation during the development of the TCESOM intoxication. However, this was not believed to be an important effect because examination of the peripheral blood did not reveal marked differences in erythrocyte counts or morphology.

Postmortem Examinations.—Gross postmortem examinations were made on the animals that died or were slaughtered. In many instances, these were supplemented by histopathological examination. While there were marked variations in the distribution and severity of the hemorrhagic lesions found in afflicted animals, the general nature and pattern of the lesions were those described by Sautter *et al.*[13]

Summary

Different specimens of trichloroethylene-extracted soybean oil meal (TCESOM) were fed at various levels to young heifers continuously or intermittently. Without exception, they induced a temporary or permanent leukopenia and thrombocytopenia. Death from aplastic anemia occurred as late as 563 days after the start of feeding 1 lb. per day of a toxic specimen.

Aplastic anemia of varying degrees of severity can be induced by feeding different levels of this meal.

Soybeans processed with trichloroethylene within a few months after harvest yielded a meal of a high degree of toxicity. Processing of old soybeans yielded a meal with a low but detectable degree of toxicity.

III. The Use of Young Calves for Study of Various Aspects of Toxicity of Trichloroethylene-Extracted Soybean Oil Meal

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Many unknown aspects of the cause and nature of aplastic anemia associated with feeding of trichloroethylene-extracted soybean oil meal (TCESOM) could not be clarified from observation of field cases.[1] A preliminary trial, carried out in May 1951 with a young dairy calf, showed that aplastic anemia could be quickly produced. Since then, young calves have been used extensively in this laboratory for screening specimens of this meal before feeding them to older animals [14] and for the development of a bio-assay method for estimating the toxic factor.[22] Picken *et al.*[3] have also adopted this technique.

Calves have been used also in a study of factors that might affect the potential toxicity of this product.

Earlier investigators reported that the feeding of trichloroethylene to cattle did not produce toxic effects.[20, 26] Others,[25] however, have maintained, without presenting evidence, that the toxicity is due to residual trichloroethylene in the extracted meal. The trichloroethylene used in extraction plants in the United States loses its stabilizer rather quickly and is subject to decomposition [5] into products, some of which may be responsible for the production of the toxic factor or might, themselves, be toxic to the cattle. For this reason, the feeding of trichloroethylene obtained from the "work tank" of one of the extraction plants was clearly desirable.

No information appears to have been published concerning the possibility that the toxic factor might also be encountered in the extractives from soybeans, i.e., in the oil or in the phosphatides which, in commercial operation, are encountered in the settlings in the oil tank referred to as "foots." Earlier investigators had suggested [26] that the phosphatides which are extensively extracted from the soybean with trichloroethylene might have a protecting effect on TCESOM intoxication.

The possible function of the rumen in the ex-

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tablishment of TCESOM toxicity has not been investigated although later studies showed that the horse, a nonruminant, also developed aplastic anemia after the feeding of this meal.[27] Among the mechanisms which have been suggested as possible causes of aplastic anemia in man, sensitivity reactions have been mentioned.[28, 29]

The susceptibility of the calf to TCESOM intoxication afforded an opportunity to test several of the factors referred to above which might be involved in the establishment of the toxicity. This paper presents a summary of pertinent observations.

MATERIALS AND METHODS

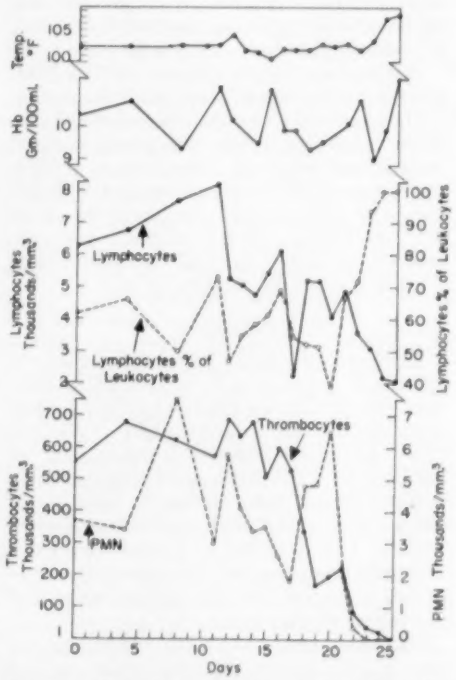
In most of these experiments, the calves weighed from 80 to 130 lb. They were obtained either from the University herd or purchased on the open market. The former group was observed for at least one week, the latter group for at least two weeks, in order to ascertain that they were essentially normal, and to obtain base line hematological values before the test materials were fed. Whole cow's milk was fed daily at a level of about 10 per cent of the body weight and the substances being tested were suspended in the milk. Clinical observations were made daily and determinations of hemoglobin, hematocrit, erythrocytes, total and differential leukocytes, and thrombocytes were made at frequent intervals by standard methods.[4] At the end of each experiment the animals were slaughtered for necropsy.

RESULTS AND DISCUSSION

Acute Toxicity of TCESOM in the Calf.—The meal was obtained from stocks remaining on a farm where cattle had died from aplastic anemia (TCESOM-1 [30]) and was fed, at a rate of 0.5 lb. per day, with milk to a healthy 70-lb. calf that had gained 8 lb. in the previous seven days. After seven days, the amount fed was increased to 1 lb. per day. Twelve days after the feeding began, the first blood changes were observed. On the thirteenth day, pyrexia and other signs of illness were observed but the animal quickly recovered and continued to eat its daily ration. On the twentieth day, bloody feces and petechial hemorrhages on the ocular, oral, and vaginal mucous membranes were observed. Subsequently, the calf also developed marked pyrexia, a bloody nasal discharge and depression which, with the bloody feces, continued until the animal died on the twenty-fourth day. It had consumed 20 lb. of the meal. Necropsy revealed hemorrhagic lesions similar in character and distribution to those previously described [13] for poisoning by this meal.

The course described above is typical of that which consistently occurred in young calves fed toxic specimens at sufficiently high levels. The temporary pyrexia, which occurred about 12 to 14 days after starting the feeding of TCESOM, is not uncommon but, in many cases, the first changes in the peripheral blood were not observed until about the 20th to 25th day. In contrast to older animals, in which the intoxication takes a more prolonged course, a decrease in the erythrocytes and the hemoglobin is usually not observed in acute intoxication of the calf. A detailed hemogram is shown (graph 1) of a calf which consumed 19 lb. of this product (0.75 lb./100.0 lb. per day).

The Effect of Various Soybean Preparations on the Young Calf.—The tolerance of young calves for soybeans or soybean oil meals had to be determined since they are seldom fed such products. Pertinent ob-



Graph 1—Changes in rectal temperature, hemoglobin, polymorphonuclear leukocytes (PMN), thrombocytes, lymphocytes, and lymphocyte percentage of calf fed milk plus 0.75 lb. of trichloroethylene-extracted soybean oil meal per day per 100 lb. of body weight. The calf died on the twenty-fifth day.

servations will be summarized without giving all details of each experiment.

Ground, raw soybeans, 0.5 lb. per day, were added to the milk ration of a healthy, 135-lb. calf which also had access to good quality alfalfa hay *ad libitum* and to a salt block containing trace minerals. A persistent diarrhea developed shortly afterwards but it could be controlled with frequent large doses of kaolin and pectin. After the tenth day, when the ration of soybeans was increased to 0.8 lb. per day, the diarrhea became progressively more marked and all attempts to control it failed. The calf began to lose weight but continued to consume its ration for 30 days. When it began to refuse some of the raw soybeans, ground soybeans, autoclaved at 15 lb. of pressure for 15 minutes, were substituted. The calf readily consumed the autoclaved beans, the ration of which was gradually increased to 1.6 lb. per day. The diarrhea soon disappeared and the calf began to gain weight. The trial was completed at the end of 90 days, after the calf had consumed the autoclaved soybeans at a rate of 1.6 lb. per day for 45 days. No signs or blood changes indicating TCESOM intoxication were observed and on necropsy no lesions characteristic of this condition were found.

A specimen * of soybean oil meal, prepared experimentally in April, 1952, by the treatment of 1951 crop soybeans with trichloroethylene in a Soxhlet-type extractor and at temperatures under 120 C.,[31] was fed to a 120-lb. calf at a level ranging from 0.5 to 0.75 lb. per day. The calf consumed 55 lb. of the meal. Diarrhea developed on the second day and continued for most of the period in spite of medication. The calf became unthrifty and developed a mild fever on the eighth day which persisted until it was slaughtered. No signs indicating TCESOM intoxication were observed, and periodic examinations revealed no blood changes. When slaughtered on the eighty-eighth day, because of general debilitation, no lesions characteristic of TCESOM were found. Autoclaved soybeans were not fed to this calf. It is not known whether the low toxicity, if any, was due to the low processing temperature, or to the interval between harvest and processing of the beans, or to both.

A specimen processed in a commercial plant from beans harvested 14 months prior to extraction (TCESOM-4[30]) was fed with milk to 2 calves for 96 days. They consumed 137 and 159 lb. of this specimen, respectively, and, judged by clinical, hematological and postmortem examinations, they remained normal. However, 2 other calves, fed a specimen (TCESOM-5) produced in the same plant on the same day from soybeans harvested only three months prior to extraction, developed typical aplastic anemia and died on the thirty-eighth and forty-fifth day, after consuming 28 and 42 lb., respectively.

Hexane-extracted soybean oil meal has been fed at levels up to 1 lb. per day to young calves and has been well tolerated. It is evident, therefore, that the blood dyscrasia and other signs of toxicity induced in young calves by certain specimens are not caused by a property inherent in soybeans but are the result of their treatment under certain conditions with trichloroethylene.

Soybean oil produced in January, 1952, in a commercial plant using the trichloroethylene process, was fed with milk to a calf weighing 127 lb. During the first 40 days, the daily intake of soybean oil was 60 Gm.; this was increased to 120 Gm. during the succeeding 50 days. Although it developed diarrhea intermittently, the calf remained healthy and continued to gain weight. No signs or blood changes suggestive of TCESOM intoxication were observed during the trial and on necropsy, no characteristic lesions were found.

The phosphatide fraction (foots), obtained from a commercial plant using the trichloroethylene process for the extraction of soybeans, was fed with milk to a healthy, 122-lb. calf. This fraction, collected during a time when freshly harvested beans were being processed, was initially fed at a level of 60 Gm. per day. The calf also had access to alfalfa hay, a salt block with trace minerals, and a small amount of grain mixture. Moderate diarrhea occurred on the second day and the feces became progressively more fluid and darker during the next six days. On the seventh day, the calf became acutely ill, weak, markedly depressed, and it appeared to be dehydrated; the rectal temperature was 106.8 F., the heart rate 160 per minute, and the blood pressure, as determined by palpation, was low. At this point, the phosphatide fraction was removed and the

* We are indebted to Dr. J. C. Cowan, U.S.D.A., for this specimen.

calf was fed by stomach tube for two days. It showed marked clinical improvement and the phosphatides were again fed, but at a level of 30 Gm. per day. When the calf continued to improve during the next seven days, 60 Gm. of phosphatide per day was fed continuously for 62 days. During the entire 71-day experimental period the calf consumed approximately 4,350 Gm. of the crude phosphatide fraction. While this was fed, diarrhea was constantly observed. The animal eventually became severely emaciated although it gained 24 lb. during the experiment. In spite of these abnormalities, no signs or blood changes typical of TCESOM intoxication were observed during the trial nor could hemorrhages be found at necropsy. It appears, therefore, that the phosphatide fraction extracted from soybeans with trichloroethylene did not contain sufficient quantities of the toxic factor to be detectable by this procedure. Cooperative studies carried out elsewhere* indicated that the addition of 1 per cent of the phosphatide fraction to a solid ration containing 50 per cent of a toxic specimen of this meal did not prevent the development of acute, fatal aplastic anemia in young calves. Likewise, the addition of the phosphatide fraction to a mixture of milk and TCESOM-1 failed to protect a young calf from intoxication.[32]

The Effect of Trichloroethylene on Young Calves.—A specimen of trichloroethylene, which had been recycled through one of the commercial extraction plants for several days, was fed to 2 calves, with milk, at an initial level of 250 mg. per day. The calves also received alfalfa hay *ad libitum* and an allowance of a grain mixture and minerals. On the 101st day, the trichloroethylene was increased for 1 calf to 10 Gm. per day while the other calf continued to receive 250 mg. per day. The latter calf, in addition, was fed 0.5 lb. of hexane-extracted soybean oil meal daily. Both animals remained normal during 346 days of feeding trichloroethylene. No blood changes were observed; at necropsy, no other abnormalities were found. The livers were normal.

Trichloroethylene, or decomposition products that may be formed from it and retained in the solvent in the commercial practice of soybean extraction, are therefore *per se* not responsible for the induction of aplastic anemia in cattle. The quantities of residual trichloroethylene found in commercial specimens are usually in the range of from 5 to 20 parts per million [33] and, therefore, are far below the amount of trichloroethylene fed in this experiment. Observations on the metabolism of trichloroethylene in the calves have been reported elsewhere.[34, 35]

Function of the Rumen in the Establishment of TCESOM Toxicity.— Although aplastic anemia had been successfully produced by feeding TCESOM to young calves in which the rumen was essentially nonfunctional, it was decided to determine whether this meal would be toxic if the rumen were by-passed. For this purpose, a fistula was established surgically * in the abomasum of a 4-week-old calf and through it a toxic specimen of this meal suspended in milk was administered at a rate of about 0.5 lb. per day. Observation with a fluoroscope revealed no detectable regurgitation from the abomasum into the rumen. This calf developed typical signs and blood changes of acute aplastic anemia 24 days after the beginning of the experiment. Necropsy revealed the characteristic hemorrhagic syndrome of TCESOM intoxication. Thus it was demonstrated that passage of the meal through the bovine rumen is not essential for the development of its toxic effects. This technique has been used successfully in more recent experiments for the administration of controlled quantities of relatively large amounts of this product to calves.[32] Control animals to which hexane-extracted soybean oil meal was administered by the same route developed normally and showed no ill effects.[32]

Is TCESOM Intoxication an Allergic Reaction?— Although evaluation of the various circumstances associated with TCESOM intoxication in cattle made it appear unlikely that an allergic response was involved, it appeared desirable to investigate this possibility. It was assumed that if the disease were an allergic reaction, the calf could be sensitized to the toxic agent by feeding a high level of this meal over a short period. At a time later than the usual induction period for TCESOM toxicity, the same animal could then be challenged with a similar quantity, and

* With Mr. C. J. Olson at Glencoe, Minn.

* We are indebted to Drs. J. P. Arnold and E. A. Usenik, School of Veterinary Medicine, University of Minnesota, for the surgical procedure.

this might induce the typical blood changes and other signs of aplastic anemia if an allergic mechanism were operative. During a pretrial period, a young 110-lb. calf was fed a ration which did not contain soybean products. Following this, the calf was fed 0.5 lb. of a highly toxic specimen of the meal twice daily for four days. No soybean products were fed during the following 37 days. From the forty-second through the forty-fifth day, this animal was again fed 0.5 lb. of the same specimen twice daily. Observation for 63 days, during which no soybean products were fed, revealed no significant blood changes or other indications of an allergic reaction. By the method used, no evidence was obtained, therefore, to support the concept that aplastic anemia might be induced through an allergic response to TCESOM.

Summary and Conclusions

The young calf is a suitable experimental animal for the study of various aspects of intoxication by trichloroethylene-extracted soybean oil meal (TCESOM).

Soybean products *per se* were found not to be toxic to calves, but specimens of the meal known to be toxic induced a fatal aplastic anemia in calves as early as 24 days after the start of feeding.

The signs and lesions of intoxication were essentially the same in calves as found in older cattle. The principal difference was that, in the calf, the erythrocyte count and the hemoglobin concentration of the blood remained essentially normal.

Trichloroethylene at levels far exceeding those present as residual trichloroethylene in TCESOM was nontoxic when fed to calves for as long as 346 days.

The toxic factor present in some specimens could not be detected in soybean oil or in the phosphatide fraction.

It was found that digestion of this meal in the rumen is not necessary for the establishment of its toxicity and that this toxicity of calves is not due to an allergic reaction.

IV. Susceptibility of the Horse to the Toxic Factor in Trichloroethylene-Extracted Soybean Oil Meal

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In conjunction with studies on the toxicity of trichloroethylene-extracted soybean oil meal (TCESOM) to cattle [14] and other species,[39,44-46] it became of particular interest to investigate the susceptibility of the horse to the toxic factor in this meal. The literature does not contain any information on this point. The syndrome of poisoning by this meal[30] in cattle, like that of poisoning by bracken fern (*Pteris aquilinum*), manifests itself as an aplastic anemia, neither of which can be cured by therapy with thiamine.[1] The horse is also susceptible to bracken poisoning but, in contrast to cattle, it responds to thiamine therapy.[53] Other species, such as the rat, which are affected by thiaminase in bracken [54] are very resistant to the toxic effects of this meal.[39] In view of the completely unknown chemical nature of the toxic factors in either bracken fern or TCESOM and the similarity of their effects on cattle, the following studies were undertaken with the horse.

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Experimental Procedure

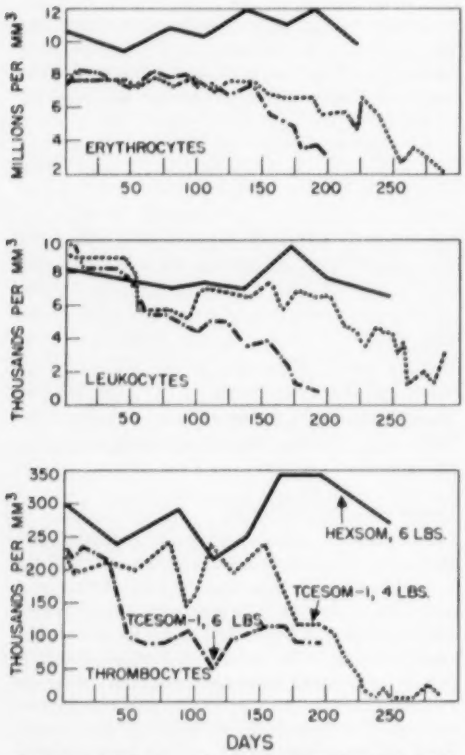
Two horses, a 14-year-old mare weighing 1,100 lb. and a 2½-year-old gelding weighing 1,000 lb., were fed a ration providing a daily intake of 6 and 4 lb., respectively, of a specimen of TCESOM of known toxicity (TCESOM-1) to cattle. The remainder of the ration consisted of good quality prairie hay, trace minerals, salt, and water *ad libitum*. As a control, a 900-lb. gelding, 3 years old, was fed daily 6 lb. of hexane-extracted soybean oil meal (purchased on the open market) and the other ingredients of the ration referred to above.

Examinations of the blood and determinations of plasma ascorbic acid, lactic acid, pyruvic acid, urea nitrogen, serum bilirubin, and thiamine in the blood were made at intervals by standard methods.[18]

RESULTS AND DISCUSSION

Clinical Observations.—The gelding fed the hexane-extracted soybean oil meal remained normal throughout the eight-month observation.

Both horses fed TCESOM consumed their entire daily allotment by the sixth day. They remained normal until the 112th day when petechial hemorrhages were found at the base of the tongue of the mare. During the next 30 days, petechial hemorrhages appeared on all of the visible mucous membranes of this animal but the only other sign of disease was oral distress, indicated by slobbering, pseudomasticatory movements, rolling of the tongue, and a hesitancy to eat scabrous roughages.



Graph 1—Effects of trichloroethylene-extracted or hexane-extracted soybean oil meal on counts of erythrocytes, leukocytes, and thrombocytes of the horse.

——— gelding fed 6 lb. of HEXSOM per day;
. . . . . gelding fed 4 lb. of TCESOM-1 per day;
– – – – mare fed 6 lb. of TCESOM-1 per day.

On the 167th day, the mare had a nasal hemorrhage, losing approximately 1 liter of blood. In the succeeding five days, other clinical signs gradually appeared until the animal had become markedly ill. The signs observed were similar to those that occur in acute TCESOM intoxication in cattle, namely anorexia, tachycardia, intermittent pyrexia with rectal temperatures varying from 99.5–107.2 F. over periods of three to ten days, serous, mucous or sanguineous nasal discharge, petechial hemorrhages on mucous membranes and thin-skinned areas, bloody feces, evidence of abdominal pain such as grunting, groaning, persistent pawing, and grinding of the teeth, and marked weakness. Many subcutaneous hemocysts appeared over the body. Numerous self-inflicted wounds bled profusely and continuously for as long as six days, even though the blood clotted on the hair surrounding the wound. This animal died after being fed TCESOM-1 for 198 days; it had consumed approximately 1,000 lb. of this feed.

No clinical signs of disease were noticed in the gelding, fed 4 lb. per day, until the 168th day when petechial hemorrhages appeared on the visible mucous membranes. The clinical syndrome was the same as in the mare, but the course of the disease was characterized by intermittent exacerbations and remissions of clinical signs of three to 20 days' duration. The animal continued to eat hay but refused to eat the meal during periods of acute illness. This animal died on the 287th day after it had consumed approximately 1,100 lb. of TCESOM-1.

Hematological Observations.—Changes in the erythrocytes, leukocytes, and thrombocytes for the 3 animals are indicated (graph 1). In the 2 horses fed TCESOM, the changes are characteristic of aplastic anemia. The first permanent blood change noticed was thrombocytopenia in the mare the fiftieth day and in the gelding the 168th day. This was followed by a progressive reduction of all cellular elements of the blood until death when both animals had severe anemia, thrombocytopenia, leukopenia, neutropenia, and a relative lymphocytosis identical with those observed in the cattle.

No hematological changes were observed in the control gelding.

*Biochemical Observations.**—A progressive reduction in the plasma ascorbic acid level in both animals fed TCESOM began on the 157th day and continued until death. The blood serum bilirubin was moderately increased in the mare just prior to her death. No deviations were noticed in the other components of the blood.

Fig. 1—Typical subcutaneous and intramuscular hemorrhages found in the mare fed 6 lb. of trichloroethylene-extracted soybean oil meal-1.

On two occasions, when the gelding was in the advanced stages of acute aplastic anemia, urine specimens were collected. The urinary carbonyl compounds were converted into 2,4-dinitrophenylhydrazine derivatives and these, after extraction, were investigated by unidimensional paper chromatography.[17] There was no apparent difference, either in the type or the amount of the 2,4-dinitrophenylhydrazine derivatives of carbonyl compounds present, in the urine from the intoxicated gelding as compared with the control animal.

* We are indebted to N. S. Mizuno for the analyses and to S. Wada for the chromatographic studies.

Pathological Changes.—Externally, fresh blood was observed to be passing from the body openings. Small hemorrhages were present on the sclera, conjunctiva, nasal, and buccal mucosa, the vagina, and other thin-skinned areas.

Numerous hemorrhages of various sizes (fig. 1) were found in the subcutaneous tissues over the entire carcass. The serosal and mucosal surfaces of the intestines and the entire peritoneum were covered with hemorrhages. Massive clots of blood were found free in the intestine. The liver was mottled with small necrotic areas. The splenic capsule was covered with hemorrhages. A few hemorrhages were observed on the capsule of the kidney and diffuse epicardial and endocardial hemorrhages were most marked over the left ventricle. Serial section of the brain revealed numerous hemorrhages throughout. In 1 horse, a large clot of blood was found beneath the dura mater at the medulla oblongata. The lungs failed to collapse and many hemorrhages were found on the pleural surfaces. The mucous membranes of the nasal passages, turbinates, trachea, and bronchi was also hemorrhagic.

Histologically, hemorrhages were widespread; the liver contained numerous foci of degeneration and necrosis and colonies of bacteria. Many of the pulmonary alveoli contained free blood and atelectasis was present in numerous areas.

Summary and Conclusions

Two horses, fed a specimen of trichloroethylene-extracted soybean oil meal (TCESOM) which was known to be highly toxic to cattle, developed fatal aplastic anemia after consuming from 1,000 to 1,100 lb. over periods of 198 and 287 days, respectively. The clinical signs, blood changes, and pathological changes were indistinguishable from those that occur in TCESOM intoxication in cattle. It is evident, therefore, that susceptibility to intoxication from this meal is not restricted to ruminants.

The feeding of as much as 6 lb. of hexane-extracted soybean oil meal per day to a gelding over an 8-month period had no harmful effects.

V. Failure to Demonstrate the Presence of a Toxic Factor in the Milk of Cows Fed Toxic Specimens of Trichloroethylene-Extracted Soybean Oil Meal

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IN EARLIER observations of Minnesota dairy herds, several cases were noticed in which calves born to cows which had consumed toxic specimens of trichloroethylene-extracted soybean oil meal (TCESOM) evidently had suffered prenatal damage.[1] This suggested that the toxic factor might have been transmitted through the placenta. It was also considered possible that the toxic factor might appear in the milk of intoxicated cows, although there was no evidence of this in the herds studied.

During an extensive field occurrence of TCESOM intoxication in Europe, in 1923 and 1925, calves were not adversely affected by consumption of milk from cows fed this meal,[26] and milk from affected cows was consumed, even by children,[27] without any apparent damage. Later, it was reported,[28] although without subsequent confirmation, from another state, where considerable intoxication, presumably from TCESOM, had occurred in cattle that several persons who had consumed milk from cows fed this meal had developed a hemorrhagic syndrome.

This paper presents a brief report on an investigation of the toxicity of the milk of cows fed toxic specimens of this meal.[30]

EXPERIMENTAL PROCEDURE

Three cows in heavy lactation were used as milk donors. They were fed either a ration (table 1) which contained a specimen of TCESOM of known toxicity, or a commercial pelleted product which had been implicated through field observations. After ten days, their milk was fed to calves, dogs, and cats (table 2) and excess quantities were pooled and frozen for later use. The calves were fed about 10 per cent of their body weight of this milk daily, also a small quantity of a concentrate mixture of crushed oats, corn, cobmeal, and alfalfa hay. The dogs were fed this milk with a mineral and vitamin mixture.[29] This was supplemented with a small quantity of horse meat for the first 45 days. The cats were fed only this milk and a mineral and vitamin supplement.[29]

Daily clinical examinations and periodic hematological examinations, as previously described,[14] were made on the donor cows and the animals receiving the milk. Necropsies were performed on all except 1 cow which was allowed to recover.

TABLE 1—Rations (lb.) Fed to Cows Daily in the Experiment to Investigate the Presence of a Toxic Factor in the Milk

Ingredient	Cow (No.)	
	958 and 959	975
Corn silage	20.0	32.0
TCESOM-1	4.8	.....
TCESOM-pellets* (average)	.....	7.2
Corn and cobmeal	1.75	3.50
Crushed oats	1.75	3.50
Linseed oilmeal	1.00	2.00
Wheat bran	1.00	2.00
Steamed bonemeal	0.06	0.11
Salt with trace minerals	0.06	0.11
Alfalfa-brome grass	*Ad libitum*	

TCESOM—trichloroethylene-extracted soybean oil meal.
* Commercial TCESOM pellets obtained from a farm where losses from acute aplastic anemia occurred in 2-year-old animals following feeding of this product. These pellets are said to contain 45 per cent TCESOM. This product was presumably used in the area mentioned in reference [28].

RESULTS

All of the cows developed blood changes and clinical signs characteristic of aplastic anemia. The data (graph 1) show that the donor cows were affected by the TCESOM during most of the time when their milk was fed to the calves, dogs, and cats.

Cow 958 developed blood changes 28 days after she was first fed the meal and 18 days after her milk was first fed to the test animals. When slaughtered, after receiving this meal ration for 62 days, her thrombocyte count was about 30 per cent and the leukocyte count about 60 per cent of the pretrial values. Necropsy revealed pathological changes characteristic of aplastic anemia.[13]

Cow 959 also developed blood changes 28 days after she was first fed the meal

TABLE 2—Animals Receiving Milk from Cows Fed Trichloroethylene-Extracted Soybean Oil Meal

Species	No.	Age or weight	Lb. milk/day	Days fed
Calf	4	100 lb.	10	91 to 96
Dog	4	3 mo.	3	90
Cat	4	2 mo.	0.4–0.5	90

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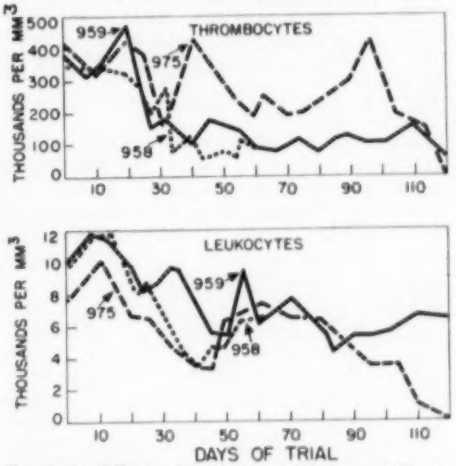
and 18 days after her milk was first fed to the test animals. The decrease of the thrombocytes and leukocytes continued during the remainder of the trial. After 106 days, when her thrombocyte count was about 20 per cent and the leukocyte count about 60 per cent of the pretrial values, she was transferred to a normal dairy ration. She then made a gradual but apparently complete recovery from the aplastic anemia.

Cow 975 developed blood changes 33 days after she was first fed the meal and 23 days after her milk was first fed to the test animals. Two days later petechial hemorrhages and other signs of incipient acute aplastic anemia were observed. To preserve her as a milk donor, she was fed a ration containing hexane-extracted soybean oil meal (HEXSOM) for six days; during this time, her thrombocyte count returned to a normal level and her clinical condition improved markedly. After again being fed the pellets containing TCESOM for 55 days, she refused to consume more pellets and became ill. She was again transferred to a ration containing HEXSOM but, after a temporary increase in the thrombocytes, she failed to make clinical improvement and died 24 days later with signs, blood changes, and lesions characteristic of aplastic anemia. Shortly before death, the thrombocyte count was 5 per cent and the leukocyte count 10 per cent of the values observed before the feeding of TCESOM.

The Effect of Milk on the Test Animals.—None of the animals which were fed milk from the affected cows showed any abnormalities while they received this milk. No information is available concerning the susceptibility of the cat to TCESOM intoxication. Other experiments [39] demonstrated that the dog is much less susceptible to intoxication from this product than the calf and that relatively high intakes over long periods are required before dogs show symptoms of toxicity. However, the calf is very sensitive to the toxic effects of TCESOM.[40] Subsequent studies have shown [22] that the daily consumption of as little as 1/40 of a pound per 100 lb. of body weight of a toxic specimen of TCESOM can induce a marked and persistent thrombocytopenia in calves in about 25 days. In this experiment the calves received milk from affected cows for a period of from 91 to 96 days; each calf consumed approximately 1,000 lb. of milk. The calves remained normal, as judged by hematological and clinical observations as well as by gross and microscopic necropsy findings, when slaughtered 91 to 96 days after the start of the experiment. If any toxic factor was secreted with the milk, it was in quantities too small to be detected by a sensitive bioassay with calves.



Graph 1—Effect of rations containing trichloroethylene-extracted soybean oil meal on leukocytes and thrombocytes of 3 milk cows.

Summary

Three heavily lactating cows, fed a ration containing a relatively high level of toxic trichloroethylene-extracted soybean oil meal (TCESOM), developed blood changes and clinical signs characteristic of aplastic anemia. Cats, dogs, and calves, fed milk produced by these cows as the principal component of their diet, failed to show clinical signs or blood changes suggestive of toxicity caused by this meal.

VI. The Effects of Feeding Trichloroethylene-Extracted Soybean Oil Meal to Sheep

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SINCE the toxic effects of trichloroethylene-extracted soybean oil meal (TCESOM) to cattle were first recognized by Stockman,[23] a few experiments have been reported on feeding this product to sheep. The results have not been uniform. Thus, Stockman [23] failed to observe signs of toxicity in a ewe fed 1 lb. per day for 42 days. Similar negative results were obtained by Eikman [41] with 2 sheep and 1 goat and at Cornell University with 8 lambs.[20] Holm *et al.*,[42] however, found that this meal which was toxic to cattle produced sickness and death in ewes and lambs with characteristic symptoms and lesions but without the typical leukopenia. Messieri [43] observed leukopenia and loose, bloody feces in a ewe fed for 62 days with 15.1 kg. of a specimen which was suspected of having caused hemorrhagic disease in cattle.

Early in our studies on aplastic anemia in cattle induced by feeding TCESOM, we investigated the possible usefulness of sheep for studies on the toxic factor in this product. The pertinent observations are summarized herewith.

MATERIALS AND METHODS

Two lambs weighing 30 lb. and 8 ewes, 4 of them pregnant, were fed rations containing a specimen of TCESOM that was known to be toxic to cattle (TCESOM-1 [20]). As controls, 2 lambs weighing 30 lb. were fed a similar ration containing hexane-extracted soybean oil meal (table 1).

Daily clinical and weekly hematological examinations were made on all animals by standard methods.[5,14]

The animals fed TCESOM received the same rations continuously until they died, except 3 ewes from group 4. These ewes, after being fed this product for 351 days, were fed a standard ration not containing the meal. Hematological examinations were continued for 72 days and clinical observations were made for 26 weeks after cessation of this feeding. The control animals were slaughtered after 78 weeks. Necropsies were performed on all animals.

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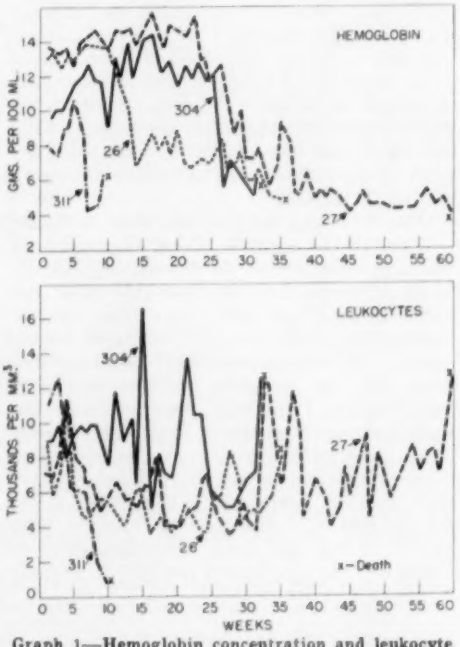
RESULTS AND DISCUSSION

Clinical Observations.—All of the sheep that were fed TCESOM developed signs of disease and all died except the 3 ewes which were transferred to a different ration after 351 days. Animal 311 died suddenly after a short clinical illness, the others after a prolonged illness. The chief signs of disease were anorexia, dullness, weakness, mucous nasal discharge, polypnea, and intermittent episodes of both pyrexia and diarrhea. The feces were often dark and blood-tinged during the period of diarrhea. Marked icterus was occasionally observed. Most animals showed repeated, marked exacerbations and remissions of the various signs and all became emaciated before death. When the first one of the lambs fed TCESOM (group 2) died, they weighed 81 and 74 lb. as compared to the



Graph 1—Hemoglobin concentration and leukocyte counts of sheep fed trichloroethylene-extracted soybean oil meal.

TABLE 1—Rations and Summary of Results of Studies with Sheep Fed Trichloroethylene-Extracted Soybean Oil Meal

Group	1	2	3	4
No. of animals	2	2	4	4
Approximate starting weight (lb.)	30	30	125	140
Reproductive status	Lambs	Lambs	Nonpregnant ewes	Pregnant ewes
Ration[1] (av. daily)				
HEXSOM[2] (lb.)	30 Gm.–1.8	.....	.....	.....
TCESOM[2] (lb.)	.....	30 Gm.–1.8	1.2–1.8	1.2–1.8
Crushed yellow corn (lb.)	30 Gm.–0.8	30 Gm.–0.8	0.6–0.9	0.6–0.9
Mixed alfalfa-brome hay (lb.)	1.0	1.0	1.0–2.0	1.0–2.0
Period until death (weeks)	[4]	38,56	10,32,32,56	18
Termination	Normal	Died	Died	Three survived 351 days
Clinical signs	None	All	All	All
Abnormal blood	None	All	All	All
Lesions	None	All	All	One

[1] Bonemeal and a salt block with trace minerals were also provided; [2] hexane-extracted soybean oil meal (HEXSOM) obtained on the open market; [3] obtained from a farm where losses had occurred when trichloroethylene-extracted soybean oil meal (TCESOM-1)[20] was fed to cattle; [4] normal when the experiment was terminated after 78 weeks.

controls which weighed 138 and 146 lb., respectively.

The 4 pregnant ewes in group 4 gave birth to 4 dead and 3 living lambs. The latter were fed a normal diet and were observed for seven to nine months; they developed normally with no signs of illness.

Hematological Observations.—The blood changes were indicative of altered hematopoietic activity and also of a hemolytic anemia (table 2). In some animals, the blood changes were characteristic of aplastic anemia and included anemia, leukopenia, neutropenia, thrombocytopenia, and an increased percentage of lymphocytes. In others, these changes were not as consistently defined but were more characteristic of a hemolytic anemia. In such animals, anemia was a fairly constant finding but the thrombocytes and the total leukocyte counts were either in the normal range or increased. The percentage of neutrophils also increased in these cases. During the four to five days prior to death, an increase in the total leukocyte count was found in all animals except No. 311.

Numerous immature forms of erythrocytes were observed in stained blood smears. The mean corpuscular hemoglobin and the mean corpuscular volume were markedly increased during the entire course of the disease. Coagulation time, clot retraction time, and prothrombin content of the blood were not changed. No

TABLE 2 — Representative Hematological Data on Sheep Fed Trichloroethylene-Extracted Soybean Oil Meal

Determination	Fed TCESOM						Fed HEXSOM	
	Signs and Symptoms							
	Aplastic anemia			Hemolytic anemia			Controls	
	Days on experiment							
	64	176	161	221	290	407	457	490
Coagulation time (min.)	5	5	5	5	5	5	5	5
Hematocrit (%)	23	19	22	20	18	14	34	39
Hemoglobin (Gm.)	5.8	4.6	6.5	6.1	5.2	3.6	11.2	13.4
Erythrocytes (x 10^{-6} per mm.^{3})	4.1	3.3	4.3	4.3	4.3	3.1	11.1	12.2
Mean corpuscular Hb. (Gm. x 10^{12})	14	14	14	14	12	12	10	11
Mean corpuscular volume (cu. μ)	56	57	51	47	42	45	30	32
Thrombocytes (x 10^{-3} per mm.^{3})	72	276	184	560	1248	536	660	556
Leukocytes (x 10^{-3} per mm.^{3})	0.7	9.7	3.6	12.9	5.05	13.0	5.75	6.5
Neutrophils (%)	1	64	49	62	59	82	37	43
Lymphocytes (%)	99	36	51	38	41	18	63	57
Erythrocyte fragility (start)	0.74	0.74	0.70	0.64	0.74	0.74	0.72	0.68
(% NaCl) (end)	0.66	0.60	0.56	0.48	0.68	0.52	0.58	0.52
Erythrocyte sedimentation rate (mm. in 7 hours)	15	18	50	60	70	75	1	1
Clot retraction (hours)	1	1	1	1	1	1	1	1
Prothrombin time (seconds) Exptl.	22	17	14	16	22	20	15	18
Prothrombin time (seconds) Control	21	17	14	15	23	20	15	18
Erythrocyte morphology	Normal			Immature erythrocytes			Normal	

HEXSOM—hexane-extracted soybean oil meal; TCESOM—trichloroethylene-extracted soybean oil meal.

consistent change occurred in the erythrocyte fragility, although these determinations showed marked variations between individuals and within the same animal at different intervals. The erythrocyte sedimentation rate in a Wintrobe hematocrit tube was 70 to 80 mm. in seven hours during periods when marked hemolysis occurred. The plasma above the cells in the Wintrobe tubes was not clear. Marked icterus was concomitant with these changes.

During part of the illness of the animals fed TCESOM, numerous small supra- and intracellular bodies having a ring, rod, or oval shape were found on the mature erythrocytes. These were believed to be *Eperythrozoon ovis*.[6] A subclinical infection with this organism was established in a lamb inoculated with blood from 1 of the infected sheep. What effect the presence of *Eperythrozoon ovis* may have had on the clinical signs and hematological changes which occurred in these animals is not clear. These bodies could not be demonstrated in the control animals which came from the same source and were housed with, and managed in the same way as, the sheep fed TCESOM.

Changes in hemoglobin and leukocytes of 4 of the animals fed this meal are shown in graph 1.

This evidence, which agrees with that reported by Holm *et al.*,[42] indicates that sheep are susceptible to the toxic effects of this meal but that they are much more resistant to the toxic effects than cattle. Thus, when this meal was fed to young heifers at a level of 1 lb. per 100 lb. of body weight, they died with typical signs of aplastic anemia in 35 to 55 days,[14] whereas sheep fed this specimen at approximately the same level of daily intake (group 3, table 1) survived for ten to 56 weeks. This, coupled with the fact that the hematological changes in the sheep are not nearly as consistent and clear-cut as those found in cattle, indicates that the sheep is not as suitable as the cow for study of the biological effects of this product and for use in bio-assay.

The pathological changes in most instances did not resemble those that occur in TCESOM intoxication in cattle. Hemorrhages of the serosal and mucosal surfaces were prominent in only 1 animal. Emaciation was the only consistent finding. Interstitial pneumonia, interstitial nephritis, enteritis, and multiple abscesses were common findings. These lesions may or may not have been secondary to the toxicity caused by this meal. Details of these examinations will be reported in another paper.

[6] Dr. E. J. Splitter, Kansas State College, Manhattan, who examined some of these specimens, confirmed this view.

SUMMARY AND CONCLUSIONS

A specimen of trichloroethylene-extracted soybean oil meal which was known to be highly toxic to cattle produced illness and signs of blood dyscrasia in sheep.

The hematological changes observed in the sheep were not always consistent with those associated with aplastic anemia.

If the same factor in this meal is responsible for the disease observed in cattle and in sheep, it appears that sheep are much more resistant to its toxic effects.

VII. Observations with Laboratory Animals Fed Trichloroethylene-Extracted Soybean Oil Meal

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SINCE Stockman [23] first associated a severe hemorrhagic disease in cattle with the feeding of trichloroethylene-extracted soybean oil meal (TCESOM), several investigators have made attempts to produce a similar syndrome in other species. To date, this has been accomplished unequivocally only with the horse.[27] In retrospect, it is not surprising that previous studies have not produced equivocal and concordant results because: (*a*) It is now evident that there are marked differences in the susceptibility of various species to TCESOM;[23, 44-46] (*b*) during earlier studies,[47, 48] the harmful effects of all types of unheated soybean meals and the need for adequate vitamin supplementation were not fully appreciated; and

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(*c*) it was not realized that the toxicity of various specimens of this product, even to a highly susceptible species, shows great differences. The latter depend, in part at least, on the interval between harvesting the beans and extraction with trichloroethylene.[20]

Unpublished experiments carried out at Cornell University, reviewed briefly by Sweeney *et al.*,[20] with rats, guinea pigs, lambs, and cattle led to the conclusion that this meal processed at temperatures of about 120 C. was not toxic. Since subsequent work [5, 20, 49] has shown clearly that this is not generally true, the validity of the conclusions drawn by some earlier investigators is subject to question. Eickman [41] did not observe toxic effects of TCESOM fed to guinea pigs, whereas Menzani [35] reported that guinea pigs were susceptible but that the clinical aspects of the intoxication in this species were different from those observed in cattle. DeGier [36] reported that it did not produce toxic effects in small laboratory animals, even though their alimentary tract was "prepared" by feeding ground glass.

To supplement the studies with cattle, calves, horses, swine, sheep, and different avian species, it was decided to reinvestigate the potential usefulness of laboratory animals for studies with this product. This paper presents a summary of pertinent observations.

TABLE 1.—Experimental Rations Fed Animals in Experiment 1 and 2

Components	Rations (Gm.) fed to: Dogs, guinea pigs, hamsters, mice, rats (exper. 1)	Rats (exper. 2)	Rabbits
Ground yellow corn	.....	.....	100
Ground oats	.....	.....	100
Alfalfa meal	.....	.....	100
Soybean oil meal	729	854	600
Molasses	.....	.....	50
Sucrose	40	.....	.....
Sucrose + vitamin mixture	100 [1]	20 [2]	10 [3]
Salt mixture	40 [3]	40 [3]	40 [3]
Hydrogenated vegetable fat (crisco)	70	.....	.....
Corn oil + vitamin mixture	20 [4]	10 [5]	.....
DL-methionine	.....	6	.....

[1] Sucrose 3,000 Gm., thiamine hydrochloride 0.12 Gm., riboflavin 0.12 Gm., pyridoxine 0.12 Gm., niacin 0.75 Gm., para aminobenzoic acid 2.25 Gm., calcium pantothenate 0.33 Gm., inositol 6.0 Gm., 2 methyl-1, 4-naphthoquinone 0.015 Gm., choline chloride 30 Gm., biotin 0.006 Gm., folic acid 0.006 Gm., and vitamin B_{12} 1.5 mg.

[2] Schultze [52] plus 40 μg. of vitamin B_{12} per kilogram of ration.

[3] Wesson [52] plus 0.013 Gm. $ZnSO_4 \cdot 7H_2$ per 100 Gm. salts.

[4] 75,000 I.U. vitamin A, 15,000 I.U. vitamin D, both as drisol ® concentrate, 25 mg. d 1-*alpha* tocopherol and corn oil to make 20 Gm.

[5] Vitamin D_3 in corn oil (200,000 I.U./Gm.) 0.750 Gm., vitamin A acetate (1 million I.U./Gm.) 1.00 Gm., d 1-*alpha* tocopherol 10 Gm., corn oil to make 1,000 Gm.

[6] Salt mixture 2, U. S. Pharmacopeia XIII.

MATERIALS AND METHODS

Expeller-type soybean oil meal (EXSOM) and hexane-extracted soybean oil meal (HEXSOM) were purchased on the open market. The TCESOM was obtained from the remaining stocks on a farm where cattle had died from acute aplastic anemia following consumption of this meal (TCESOM-1[20]). During the last three months of the experiment with dogs, another specimen was used which was also known to be toxic to cattle (TCESOM-5[20]).

The composition of the rations fed to the various species in different experiments is shown in table 1. Table 2 presents a summary of the numbers of the animals fed the various rations and indicates the control animals used.

The weights of the animals at the start of the different experiments were as follows: guinea pigs 135 to 201 Gm.; albino rats (exper. 1) 135 to 142 Gm.; black rats, all female, (exper. 2) 29 to 47 Gm.; mice 10 to 14 Gm.; hamsters (adult) 172 to 194 Gm.; rabbits 1,250–2,900 Gm.; dogs 20 to 33 lb. The rats, guinea pigs, rabbits, and hamsters were housed in metal cages with wire floors; the mice in standard mouse boxes with wood shavings for litter. All animals were kept in rooms maintained at approximately 80 F. Feed and water were given *ad libitum*. In addition, each guinea pig received a daily injection of 2 to 5 mg. of sodium ascorbate intramuscularly or subcutaneously.

All animals were inspected daily and weighed at frequent intervals. Blood for hematological examination was obtained periodically from the marginal ear veins of the rabbits, guinea pigs, and hamsters, from a cut portion of the tail of rats and mice, and from the radial vein of the dogs. Hematological determinations by standard methods [6] included hemoglobin and total and differential leukocyte counts in the rats (experiment 1 only), mice, and hamsters. In addition, erythrocyte and total leukocyte counts were made in rats of experiment 2 and thrombocyte counts and coagulation times were determined in the guinea pigs; the hematological studies with the dogs included all of the determinations indicated above plus hematocrit and erythrocyte sedimentation rate. Determinations of blood urea [9] were made on the dogs and some of the guinea pigs during the later stages of intoxication.

Necropsies were performed on all animals at the end of the study and, in many instances, tissues were subjected to histopathological examinations.

After it became apparent that the rats in experiment 1 were not adversely affected by TCESOM, experiment 2 with rats was started. It was believed that the feeding of this meal at a still higher level to weanling rats of a different strain might reveal signs of susceptibility. In addition, representative rats from experiment 2 were subjected to two types of stresses as follows:

1) Phenylhydrazine Anemia.—Five rats each were selected from the groups which had been

fed either the TCESOM or the HEXSOM diet for about nine months. Base-line values for erythrocytes and leukocytes were established by three consecutive counts. The 10 rats were then injected intraperitoneally with a 0.5 per cent aqueous solution of recrystallized phenylhydrazine hydrochloride at a level of 0.9 mg. per 100 Gm. of body weight. Fifteen days later, the injection of the same dosage was repeated. Erythrocyte and leukocyte counts were made every one to two days after each injection. Two other groups of 5 rats each, fed TCESOM or HEXSOM, respectively, were injected intraperitoneally with a 0.5 per cent solution of recrystallized phenylhydrazine hydrochloride at a level of 6 mg. per 100 Gm. of body weight. This injection was repeated 14 and 26 days after the first injection. Periodic blood counts were made before and after each injection.

2) Induced Hyperthyroidism.—Into the diet of two groups of 5 rats each, which had been fed 85.4 per cent of TCESOM or of HEXSOM, respectively, for nine months, iodinated casein (protamone)* was incorporated. The level of iodinated casein was gradually increased, during a ten-day period, from 2 per cent to 4 per cent, where it was maintained until the end of the experiment. Into the ration of two other groups of 15 rats each, iodinated casein was introduced at a constant level of 2.5 per cent for 30 days.

* We are indebted to Dr. George O. Kohler, Cerophyl Laboratories, Inc., Kansas City, Mo., for a generous gift of this product.

RESULTS AND DISCUSSION

Guinea Pigs. — All of the guinea pigs fed TCESOM developed a chronic wasting disease (fig. 1) and died after 35 to 125 days. The most prominent clinical signs were emaciation, unthriftiness, and diarrhea. Alopecia occurred in some animals while the hair of others became long and shaggy. Early in the clinical course, hyperesthesia was commonly observed while later many animals became stuporous. Polydipsia was common during the latter part of the clinical course. No elevation of body temperature or hemorrhages were seen in these animals. All of the guinea pigs fed EXSOM, HEXSOM (fig. 2), or the commercial guinea pig diet remained healthy during the 137-day experiment and made comparable weight gains.

Examinations revealed few marked differences in blood components of the animals fed HEXSOM, EXSOM, or TCESOM. There was no evidence of reduced hematopoietic activity. During periods when advanced clinical signs were present, however, many afflicted animals fed TCESOM had leukocyte counts that were somewhat higher than those of the control animals, and the blood urea was abnormally high

TABLE 2—Results of Feeding Experimental Rations to Laboratory Animals

Species	No. of animals	Type of soybean oil meal	Supplement	Symptoms	Blood changes	Termination	
						Cause [4]	Days
Guinea pig	10	EXSOM	.....	None	None	K	125–137
Guinea pig	4	HEXSOM	.....	None	None	K	137
Guinea pig	37	TCESOM	.....	All	None	D	58–125
Guinea pig	6	TCESOM	0.5% DL-methionine	All	None	D	68–90
Guinea pig	7	TCESOM	1% phosphatides	All	None	D	35–123
Guinea pig	5	Commercial [1]	.....	None	None	K	110–120
Guinea pig	4	Nonheated TCESOM	.....	All	None	D	15–34
Rat, Exper. 1	6	EXSOM	.....	None	None	K	495
Rat	10	TCESOM	.....	None	None	K	180–495
Rat	6	TCESOM	0.5% DL-methionine	None	None	K	154
Rat	4	TCESOM [2]	.....	None	None	K	180
Rat	7	TCESOM [3]	.....	None	None	K	192
Rat, Exper. 2	34	HEXSOM	.....	None	None	ST	273
Rat	35	TCESOM	.....	None	None	ST	273
Mouse	8	EXSOM	.....	None	None	K	90
Mouse	8	HEXSOM	.....	None	None	K	90
Mouse	36	TCESOM	.....	None	None	K	30–90
Mouse	8	TCESOM	0.5% DL-methionine	None	None	K	90
Rabbit	10	TCESOM	.....	None	None	K	68–113
Rabbit	1	Pellets [5]	.....	None	None	K	82
Hamster	3	TCESOM	.....	None	None	D	156–207
Dog	3	HEXSOM	.....	None	None	K	500
Dog	4	TCESOM	.....	All	All	D	378–483
Dog	4	HEXSOM	.....	None	None	K	180

[1] A commercial guinea pig ration obtained on the open market; [2] 60% TCESOM and 40% commercial ration; [3] commercial ration presumably containing 45% TCESOM; [4] K—killed; D—died; ST—used for stress experiments; [5] commercial rabbit pellets not containing TCESOM.

TCESOM—trichloroethylene-extracted soybean oil meal; EXSOM—expeller-type soybean oil meal; HEXSOM—hexane-extracted soybean oil meal.

Fig. 1—Guinea pig fed ration containing 72.9 per cent of trichloroethylene-extracted soybean oil meal for 100 days.

Fig. 2—Guinea pig fed ration containing 72.9 per cent of hexane-extracted soybean oil meal for 100 days.

(50 to 169 mg./100 ml. of blood) in comparison with the control animals (16 to 34 mg.).

The most prominent pathological changes in guinea pigs fed TCESOM were degeneration of the renal tubules, severe emaciation, and gastric ulceration with hemorrhage into the digestive tract. No lesions similar to those that occur in TCESOM intoxication in cattle were encountered. No abnormalities were found in animals fed EXSOM, HEXSOM, or the commercial rations.

Four guinea pigs were fed TCESOM* produced in the laboratory by extraction of soybean flakes in a Soxhlet-type extractor at a temperature of less than 120 C.[40] All of these animals developed marked diarrhea and lost weight rapidly and all of them died after 15 to 34 days. Necropsy revealed emaciated carcasses and a few small hemorrhages scattered throughout the body.

Supplementation of the ration containing 72.9 per cent of TCESOM-1 with 0.5 per cent DL-methionine or with 1 per cent soybean phosphatides did not detectably alter the course of the disease in guinea pigs.

Rabbits.—Six of the rabbits, kept in groups of 2, were fed restricted amounts (44 to 53 Gm. per animal per day) of a commercial rabbit chow and TCESOM *ad libitum*, of which they consumed from 11 to 30 Gm. per day. For the 2 smallest rabbits, only 20 per cent of their total food intake was the meal; for the other 4 rabbits, the consumption of this meal averaged about 35 per cent of the total food intake. Four rabbits were fed the ration (table 1) containing 60 per cent of the meal. The average rate of gain was: for the 2 older, heavier rabbits, 4.2 Gm. per day; for the younger, lighter rabbits, 9.7 to 12.5 Gm. per day; for the control rabbits fed no TCESOM, 11.7 Gm. No signs of toxicity were observed throughout the experiments which lasted for various rabbits from 68 to 113 days. The total and differential leukocyte counts and thrombocyte counts were in the range of values normally found in rabbits of similar ages. At necropsy, no abnormalities were found. These observations are in accord with those of Willeke.[50]

Hamsters.—None of the 3 hamsters developed signs of disease or blood changes, although they gradually became emaciated and died 156, 184, and 207 days, respectively, after the start of the experiment. No pathological changes were observed at necropsy. There was not sufficient evidence to indicate whether the death of these animals was associated with the meal intoxication, some other cause, or factors associated with old age. It was evident, however, that the consumption of high levels of this meal did not induce signs of aplastic anemia in this species.

Mice.—None of the mice fed the 72.9 per cent soybean oil meal rations for periods to 90 days developed signs or blood changes indicative of disease and no pathological changes were found at necropsy. Six female mice that had consumed TCESOM for 45 to 58 days were bred by males maintained on the same ration for a like period. All of these females became pregnant and had litters of 6 to 11 young. Some of the F_1 generation of mice were maintained on

* This specimen was made available by Dr. J. C. Cowan, Northern Regional Utilization Branch, U.S.D.A., Peoria, Ill.

Fig. 3—Dog fed ration containing 72.9 per cent of trichloroethylene-extracted soybean oil meal for one year.

the TCESOM diet until they were 64 days old but none developed signs of disease.

Dogs.—The dogs in both groups were healthy and gained weight for the first two months of the experiment, but at that time those fed the TCESOM diet began to lose weight and were not as alert and active as the others. During the following two to three months, these dogs gradually became emaciated and were generally depressed. Fever and diarrhea also occurred intermittently. The dogs died after consuming this meal for 54 to 69 weeks. The final clinical stages of the illness were characterized by marked emaciation, dehydration, extreme weakness, inappetance, and polyuria (fig. 3).

Hematological examinations revealed marked variations in the results of all determinations. During some periods of the clinical course, changes suggestive of reduced hematopoietic activity such as thrombocytopenia, leukopenia, and a relative lymphocytosis were noted. These periods, however, were always short and were followed by wide fluctuations in the thrombocyte and leukocyte counts. Sharply defined changes characteristic of aplastic anemia were not observed in any of the dogs fed TCESOM. Blood urea nitrogen levels tended to be high in all animals fed this product. Gross pathological examination revealed marked emaciation and a few petechial hemorrhages but no other pronounced changes.

In contrast to this, the control dogs fed HEXSOM remained healthy, with no cellular or biochemical blood changes. One of the control females, bred by a male fed the same ration, whelped a normal litter. Four additional bitches were fed the HEXSOM diet for six months; during this time, they were bred and whelped and raised normal litters.

Rats.—Experiment 1.—None of the rats fed TCESOM or EXSOM developed signs of disease or blood changes and no pathological changes were found at necropsy. The rats fed either of the 72.9 per cent soybean oil meal rations did not grow as rapidly or reach as great a maximum weight as those fed the rations containing 45 and 60 per cent TCESOM. Supplementation of the rations with 0.5 per cent of DL-methionine did not alter the growth response of the animals fed the high TCESOM diet.

Experiment 2.—Weight Gains.—During the first six and 12 weeks, the group fed HEXSOM gained an average of $99.3 \pm 1.6^*$ Gm. and $128.2 \pm 1.96^*$ Gm., respectively, while those fed TCESOM gained an average of $81.4 \pm 2.20^*$ Gm. and $113.3 \pm 1.65^*$ Gm., respectively. The probability that this difference is due to chance is less than 0.0001. The difference in weight gain became apparent during the first few days, and it is correlated with the fact that the rats fed TCESOM consumed during the first five weeks only 65 to 80 per cent of the amount of feed eaten by those fed HEXSOM. Afterward, the feed consumption of the two groups and the rate of weight gain were not significantly different. Thus, between the sixth and the twelfth week, the group fed TCESOM gained an average of $31.7 \pm 1.36^*$ Gm.; those fed HEXSOM, an average of $28.8 \pm 1.29^*$ Gm. The probability that this difference is due to chance is 0.13; hence, the difference is not significant.

Intraperitoneal Injections of Phenylhydrazine Hydrochloride.—After the first injection of 0.9 mg. of this compound per 100 Gm. of body weight, there was in both groups (5 rats on each feed) an equal and temporary decrease in body weight and food consumption. Ten days later, however, the pretreatment levels of body weight and food consumption were reached again. The second injection of the same relative amount of phenylhydrazine hydrochloride, 15 days after the first, had no effect on either food consumption or body weight. After the first injection, the erythrocytes in both groups decreased equally in five days, from 9 million to 4 million per cubic

* Standard error of the mean.

millimeter; on the fifteenth day when the second injection was given, the erythrocyte count had reached, in both groups, about 5.6 million. It was not further depressed by the second injection and it continued in both groups to increase gradually at an equal rate to a level of 9 million, which was reached 50 days after the first injection. The leukocyte count remained essentially the same in both groups after both injections although 2 rats in each group showed a temporary leukocytosis after each injection.

In the other two groups of 5 rats, each of three injections of 6.0 mg. of phenylhydrazine hydrochloride per 100 Gm. of body weight produced, in both groups, a temporary and equal decrease in food consumption and in body weight. After each of the three injections, the rats in both groups developed a temporary anemia with a drop in erythrocyte counts, within three to four days, of 5 million per cubic millimeter after the first injection and of about 4 million after the second and third injections. In each instance, however, the erythrocyte counts in both groups increased by about 4 million ten days after reaching the minimum. After each injection, the rats fed HEXSOM developed a marked temporary leukocytosis with a mean increase, within three to four days, of 23,000, 18,000, and 8,000 leukocytes per cubic millimeter following the first, second, and third injections, respectively. Within eight days after each injection, the leukocyte count decreased to about the preinjection level. The rats fed TCESOM produced a leukocytosis after the first and second but not after the third injection of phenylhydrazine hydrochloride at this level. The mean increases in leukocyte counts in this group were about 14,000, 4,500, and 0 per cubic millimeter, respectively, after the first, second, and third injections.

While the number of animals thus tested was small and the individual variations relatively large, the results suggest the possibility that the capacity of the rat, fed TCESOM at high levels, to respond to repeated leukopoietic stimuli may be impaired. This point may merit further investigation with preparations of this meal or concentrates of higher potency.

The Effect of Induced Hyperthyroidism.—When iodinated casein was fed at a constant level of 2.5 per cent of the diet or in amounts increasing gradually from 2 to 4 per cent during a ten-day interval, the rats fed either TCESOM or HEXSOM lost weight at about an equal rate and they died in from 15 to 30 days. The two groups of rats, therefore, appeared to be equally susceptible to the stress of induced hyperthyroidism.

Summary and Conclusion

A specimen of trichlorethylene-extracted soybean oil meal (TCESOM), which is highly toxic to cattle, was fed for long periods at high levels to mice, rats, hamsters, guinea pigs, rabbits, and dogs.

In contrast to cattle and horses, none of these species showed any evidence of aplastic anemia or other marked blood dyscrasia.

Feeding of this meal to guinea pigs invariably produced a syndrome of gradual generalized debilitation which terminated in death. Guinea pigs fed similar quantities of HEXSOM gained weight and remained normal.

Feeding of high levels of TCESOM to dogs induced the gradual development of anorexia, weight loss, and a generalized debilitation. The dogs ultimately died. Feeding of the same amount of HEXSOM to dogs had no detectable harmful effects.

It is not known whether the factor in TCESOM which produces debilitation and death in guinea pigs and dogs is the same as that which is responsible for the production of aplastic anemia in cattle and in the horse.

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Research on Foot-and-Mouth Disease. X. The Titration of Cultivated Foot-and-Mouth Disease Virus (Frenkel) in Cattle Compared with the Titer Obtained in the Quantitative Complement-Fixation Test

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IN THE preparation of foot-and-mouth disease vaccine, the most important single item is the knowledge of the concentration of virus to be used in the vaccine.

In most foot-and-mouth disease laboratories, the method of virus titration used is that described by Henderson.[1] Two or more cattle are necessary for each titration. After anesthetizing the animals, the tongue is easily drawn from the mouth and its epithelial surface divided into several parts by making intradermal injections of India ink transverse to the long axis of the dorsal surface. In front of the marking line, 10 intradermal injections of the highest dilution of the virus suspension are made in the quantity of 0.1 ml. to 0.2 ml. per injection and behind this line, 10 injections of the lowest dilution are made. This is done in order to eliminate the influence of the lowest dilution on the highest. In this laboratory, the dilutions of 10^{-6} and 10^{-5} are routinely used.

The body temperatures of the animals are recorded before the injections of virus suspensions are made and also at 21, 24, 27, and 30 hours after these injections. Thirty hours after inoculation, the surface of the tongue must be inspected for specific lesions. This examination is the final one before the animals are slaughtered.

The infectious dose $(i.d.)_{50}$ end points of the virus suspension can be calculated by the method of Reed and Muench.[2] This method of titration is highly accurate, but very expensive for a country like Holland because it is necessary to import animals from a country free of foot-and-mouth disease, e.g., Ireland. Native animals can not be used because 95 per cent of the cattle population has been vaccinated. It has been observed that these cattle do not show reactions after injection with a virus suspension that had an end point of 900,000 $i.d._{50}$ when tested on Irish animals.

Special measures of isolation are required in order to prevent contamination of the test animals with foot-and-mouth disease virus. It was because of these factors that we have studied the correlation between the complement-fixing ability and the infectivity of this virus. The virus employed had been cultivated by the method of Frenkel and routinely used in this laboratory for vaccine production.[3-6]

Traub and Pyl,[7] Bachrach,[8] and Bradish *et al.*[9] described methods of ultracentrifugation for separation of the strict complement-fixing components from the component which is concerned with both infectivity and complement fixation. These components are not completely identical. That there is a correlation between them was established by means of the complement-fixation test. Therefore, it is possible to prepare satisfactory vaccines from a given virus culture by using the amount of complement fixed by the combination of antigen and homologous serum as a guide. This in turn can be used as an index of the concentration of antigen available in the culture for vaccine preparation.

To make this determination, it is necessary to standardize all components of the test and to ascertain the quantity of complement fixed by measuring the degree of hemolysis of a given erythrocyte suspension. The Fisher electrophotometer is used to measure the hemolysis. In this apparatus, tubes of 5-ml. volume and filter No. 425B (approximate wave length 4250 A) are used.

To check the electrophotometer and to check the relationship existing between the data indicated on the scale of the meter and the percentage of hemolysis, we composed an extinction curve on linear graph paper of known hemoglobin concentrations. We plotted the percentage of hemolysis on the abcissa and the extinction on the ordinate (graph 1). For the preparation of the hemoglobin concentrations *see* under materials and methods.

MATERIALS AND METHODS

As a diluent, 0.85 per cent saline was employed in all tests.

The Sheep Red Blood Cells.—A 2.5 per cent suspension of cells was prepared from sheep blood

From the State Veterinary Research Institute, Amsterdam, Dr. H. S. Frenkel, director.

that was preserved in an equal volume of modified Alsever's solution. After washing and centrifugation, 0.1 ml. of the packed cells was added to 4 ml. of saline. To 0.8 ml. of that mixture, we added 3.2 ml. of distilled water in order to produce complete hemolysis. In the test, the extinction must be 80 (100 % hemolysis).

TABLE 1—Percentages of Hemolysis when Different Concentrations of Hemoglobin and Saline Are Employed

Hemoglobin concentrations (ml.)	Hemolysis (%)	Saline (ml.)	Extinction
0.0	0.0	4.00	0.0
0.02	2.5	3.98	5.0
0.40	50.0	3.60	50.0
0.80	100.0	3.20	80.0

Corrections to this reading are made by adding or removing saline. The final volume can be determined according to the formula:

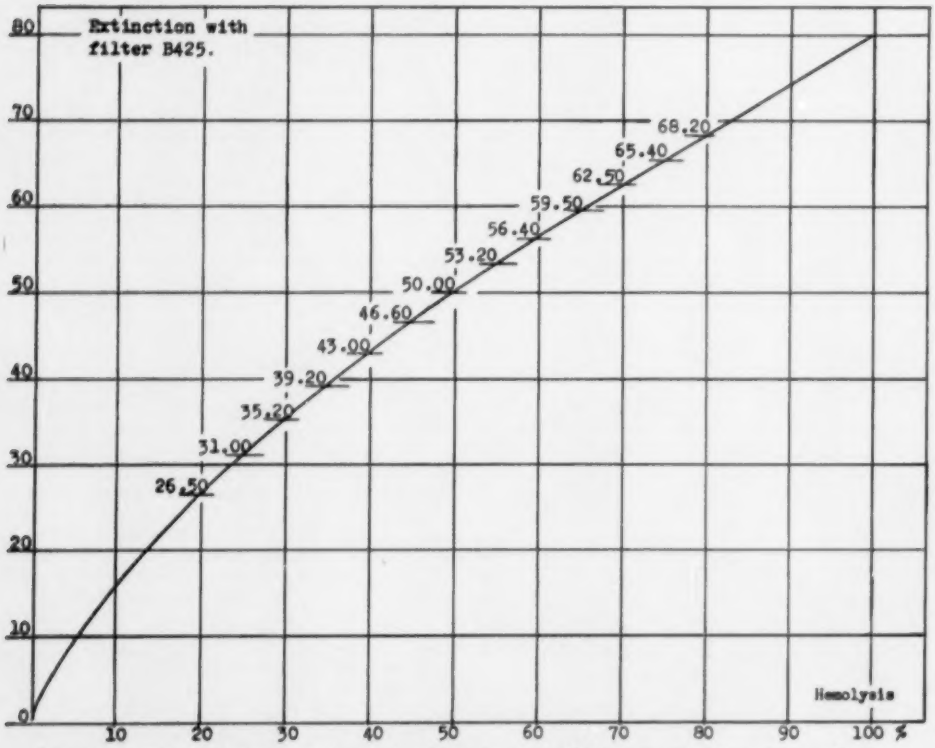
$$V_s = \frac{VI \cdot E}{80}$$

where VI is the volume of the suspension yielding extinction E and V_s the final volume to yield an extinction of 80. If the extinction is more than 80, it is necessary to add saline; less than 80, a part of the blood suspension must be centrifuged again and some of the saline removed. This procedure which is used by the medical corps of the United States Army to standardize a blood suspension was developed by Kent, Bukantz, and Rein.[10, 11]

The Standard Hemoglobin Concentrations.—A standard hemoglobin concentration can be prepared by adding 0.1 ml. of packed cells to 4.0 ml. of distilled water. The extinction of 0.8 ml. of this concentration with 3.2 ml. of saline must be 80. By taking different quantities of this concentration and adding saline until the total volume is 4.0 ml., it is possible to change the percentage of hemolysis (table 1).

In this manner, hemoglobin solutions with a percentage of hemoglobin ranging from 0 to 100 per cent were prepared and determined by the extinction for each percentage.

The Amboceptor.—The amboceptor is prepared by injecting rabbits which are in excellent nutritional condition. A series of intravenous injections with different amounts of washed sheep red blood



Graph 1—The percentage of hemolysis of known hemoglobin concentrations composed on extinction curve.

cells were made; 0.3 ml. was given on the first and third day, 0.6 ml. on the fifth day, and 1.0 ml. on the eighth, twelfth, and fifteenth day. Two days after the last injection, the rabbits are destroyed and the blood collected. It is placed for 15 minutes in an incubator at 37 C. and then for a half hour in the refrigerator at 4 C. The blood must be centrifuged for ten minutes at 3,000 r.p.m. The supernatant serum is poured into a calibrated flask and sufficient 5 per cent phenol is added to make a final concentration of 0.5 per cent. The complement present in the serum is destroyed by the phenol solution after one week and the amboceptor can be titrated. It can be kept in the refrigerator for years without any decrease in titer.

The Titration of the Amboceptor.—Before using the electrophotometer, we expressed the degree of hemolysis as follows: ++++ = 100 per cent fixation; +++ = 75 to 100 per cent fixation; ++ = 50 to 75 per cent fixation; + = 25 to 50 per cent fixation; — = no fixation.

From our amboceptor, we prepared dilutions of 1:2,000 to 1:15,000. To 0.5 ml. of each dilution was added 0.5 ml. of a 10 per cent complement dilution and 0.5 ml. of a 2 per cent erythrocyte suspension. After incubating for a half hour at 37 C., the readings were made. The highest dilution with a maximum hemolysis was considered to be the titer of our amboceptor. Usually it was 1:12,000. It was then used in the dilution 1:3,000 in order to have a surplus of amboceptor. This method is very accurate.

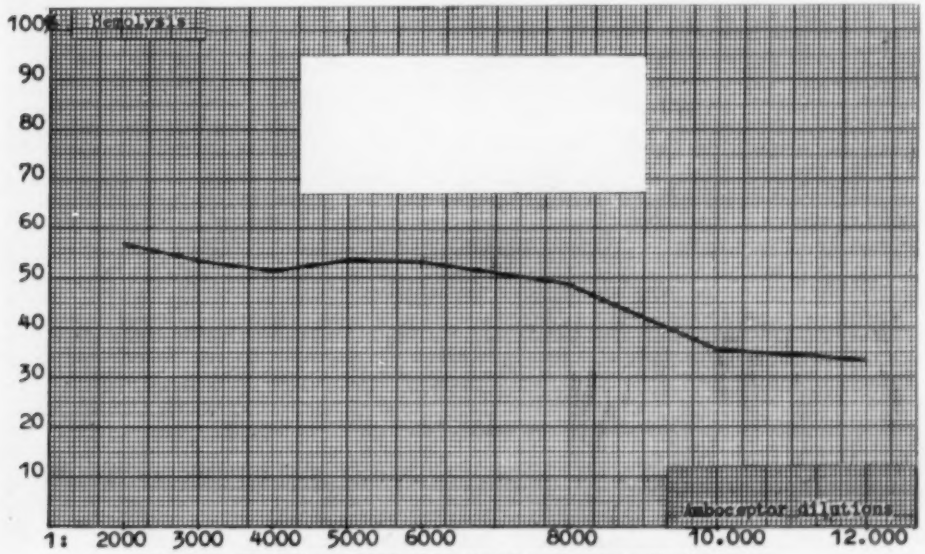
Kent *et al.*[10] described a method of determining the optimal amboceptor dilution by estimating the unit of complement giving 50 per cent hemolysis in a previous titration with a surplus of amboceptor. Using that unit, they determined the capacity for hemolysis of blood cell suspensions which were sensitized with different amounts of amboceptor. This was done in order to determine whether the hemolytic property of the blood cells will change with the use of different dilutions of amboceptor.

With the data obtained they estimated, by using the von Krogh[12] formula (for the application of that formula *see* titrations of the complement), the different units of complement for each amboceptor dilution. The values of these units were plotted against the amboceptor dilutions. The unit did not change very much. Knowing that there was a surplus of amboceptor when the dilution was 1:3,000, we determined the unit of complement (*see* section: complement titration).

Using this unit with different dilutions of amboceptor (1:2,000 to 1:12,000) the optimal dilution was determined, *i.e.* that dilution beyond which any decrease in concentration fails to diminish appreciably the unit of complement. The amboceptor dilutions were plotted on graph paper against the extinction values. A smooth line can be fitted to the resultant co-ordinate points (graph 2).

In the dilution lower than 1:2,000, agglutination of the blood cells occurred. In the dilutions higher than 1:8,000, the unit of complement does not give 50 per cent hemolysis. In all our tests, the dilution of 1:4,000 was used.

Complement Titration and Determination of the Unit.—The complement used is pooled guinea pig serum mixed with an equal part of Witte's solution containing boric acid, 2 Gm., potassium sulphate, 5 Gm., and sufficient distilled water to make 50 ml.[11] With this preservative, complement can



Graph 2—The amboceptor dilutions plotted against the extinction values.

TABLE 2—Example for Estimating the Unit of Complement

Physiol. salt solution (ml.)	Complement 1 : 50 (ml.)	Hemolytic system (ml.)	Extinction	Hemolysis (%)	K's quantities to give 50 % hemolysis (ml.)
2.10	0.30	1.60	14.5	.....	.....
2.05	0.35	1.60	21.0	.....	.....
2.00	0.40	1.60	30.0	24.00	0.5084
1.95	0.45	1.60	38.0	33.50	0.5189
1.90	0.50	1.60	50.0	50.00	0.5000
1.85	0.55	1.60	56.0	59.25	0.5090
1.80	0.60	1.60	64.0	72.50	0.4907
1.75	0.65	1.60	69.5	.....	.....
1.70	0.70	1.60	71.0	.....	.....
1.65	0.75	1.60	78.0	.....	.....
1.60	0.80	1.60	80.0	100.00	.....

In this titration the amount of complement which has been used is 2.5270 ml. for 5 tubes. The average K for 1 tube is 2.5270 ml. ÷ 5 = 0.505 ml.

The determination of the unit is only necessary for the titration of amboceptor and to control a new batch of complement.

be kept in the refrigerator for six to seven weeks without a significant loss of titer.[12]

A stock dilution of 1:50 is prepared by adding 1 ml. of complement to 49 ml. of saline. The titration of the complement is carried out as described (table 2). The red blood cells are sensitized for 30 minutes at 37 C. with the amboceptor dilution of 1:4,000 and then added to the different quantities of complement. The mixture must be well shaken. After an incubation of 30 minutes, the tubes are centrifuged for five minutes at 2,000 r.p.m. and the extinction determined.

The relation between extinction and the percentage of hemolysis is known from graph 1.

Using the von Krogh formula:[13]

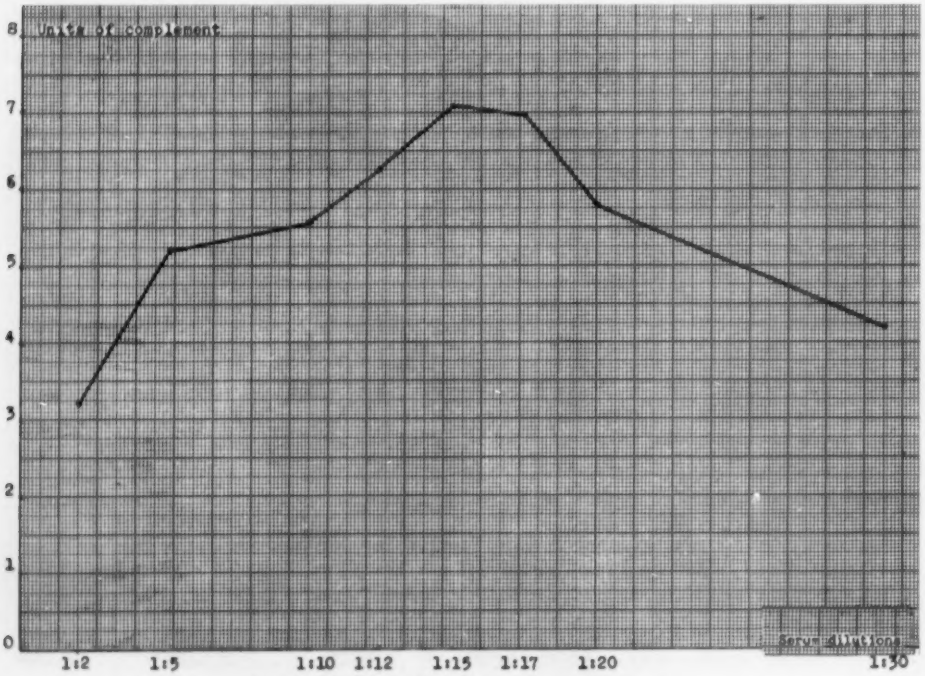
$$\log x = \log K + 1/n \quad \frac{y}{1-y}$$

the values of 1/n and K can be estimated. In that formula:

x = the quantity of complement used,
K = a constant, the quantity of complement necessary for 50 per cent hemolysis,
1/n = a constant, representing the slope of the nearly straight line obtained by plotting log x as ordinate against log $\frac{y}{1-y}$ as abscissa.

The numerical value of 1/n may be estimated by the graphical method, or mathematically, by using the method of least squares. We used the latter and estimated from the results of 25 experiments the value of 1/n as 0.207. Any value for K, expressing the quantity of complement required for 50 per cent hemolysis, can be calculated in accordance with the relation:

$$\log K = \log x - 1/n \cdot \log \frac{y}{1-y}$$



Graph 3—The titration of type O serum, tube 6.

The constant value of 0.207 is substituted for 1/n; for x, the amounts of complement from 0.30 ml. to 0.90 ml. with intervals of 0.05 ml.; for y, values with the range of 20 to 80 per cent with intervals of 0.25 per cent. For each value of these substitutions, the value of K has been calculated and the various values placed in a card file, thus it is no longer necessary to solve the von Krogh formula mathematically, but only to refer to the cards.

An example for estimating the unit of complement is shown (table 2).

Preparation and Titration of the Hyperimmune Serum.—Cattle strains of virus which are adapted to the guinea pig are injected intradermally in both pads of guinea pigs. After a convalescence of six weeks, the animals are injected three times intramuscularly with an interval of two to four days using 0.2 ml. of a 10 per cent suspension of freshly infected guinea pig pads. After these, two injections in the saphenous vein are given, the first one being 0.5 ml. of a 10 per cent suspension, the second two to four days later being 0.5 ml. of a 20 per cent suspension. For ease of injection, it is necessary to anesthetize the animals. They are destroyed two to four days after the last injection and their blood collected. Serums of at least 10 guinea pigs are pooled. To preserve the serum, merthiolate solution (1:1,000) is added to make a final concentration of 1:10,000. The serum is stored at —30 C.

In the titrations of serum and antigen, the complement is incubated twice. To eliminate the influence of the antigen, and also changes in the complement by the second incubation, the unit of complement has to be estimated in the presence of antigen and serum. Normal guinea pig serum inactivated for a half hour at 56 C. is used.

Each serum dilution, which must be used in order to determine the optimal dilution, needs its "own" unit of complement which must be calculated by adding the normal serum in the same dilution.

From previous titrations on oxen, we have noticed that the average $l.d._{50}$ titer for type A cultures was 1:700,000; for type O cultures, 1:900,000; and for type C cultures, 1:400,000. Cultures with these titers were used as antigen in the serum titrations.

After the usual incubation periods and centrifugation to sediment the red cells, electrophotometer readings are made.

In the tube containing the greatest amount of complement (tube 15), there is 100 per cent hemolysis (table 3). This should be equal to 80 on the meter, but because of the presence of the serum and the antigen the scale usually indicates more than 80. With the knob marked "initial null" the instrument must be adjusted to 80 in order to eliminate the influence of the color of serum and antigen. The 50 per cent hemolytic dose of complement for every serum dilution is then calculated. The dilution fixing the greatest amount of complement (in the example given 1:15 using 7.1 units of complement) is the optimal one (graph 3).

In graph 3, a "zone effect" can be distinguished in the dilutions 1:2 up to 1:15. This is due to thermostable complement components of the hyperimmune serum. These enlarge the total quantity of complement components in the entire system.

The advantage of using culture virus as antigen rather than cattle virus is that the first never shows an anticomplementary effect and that it can easily be standardized.

RESULTS

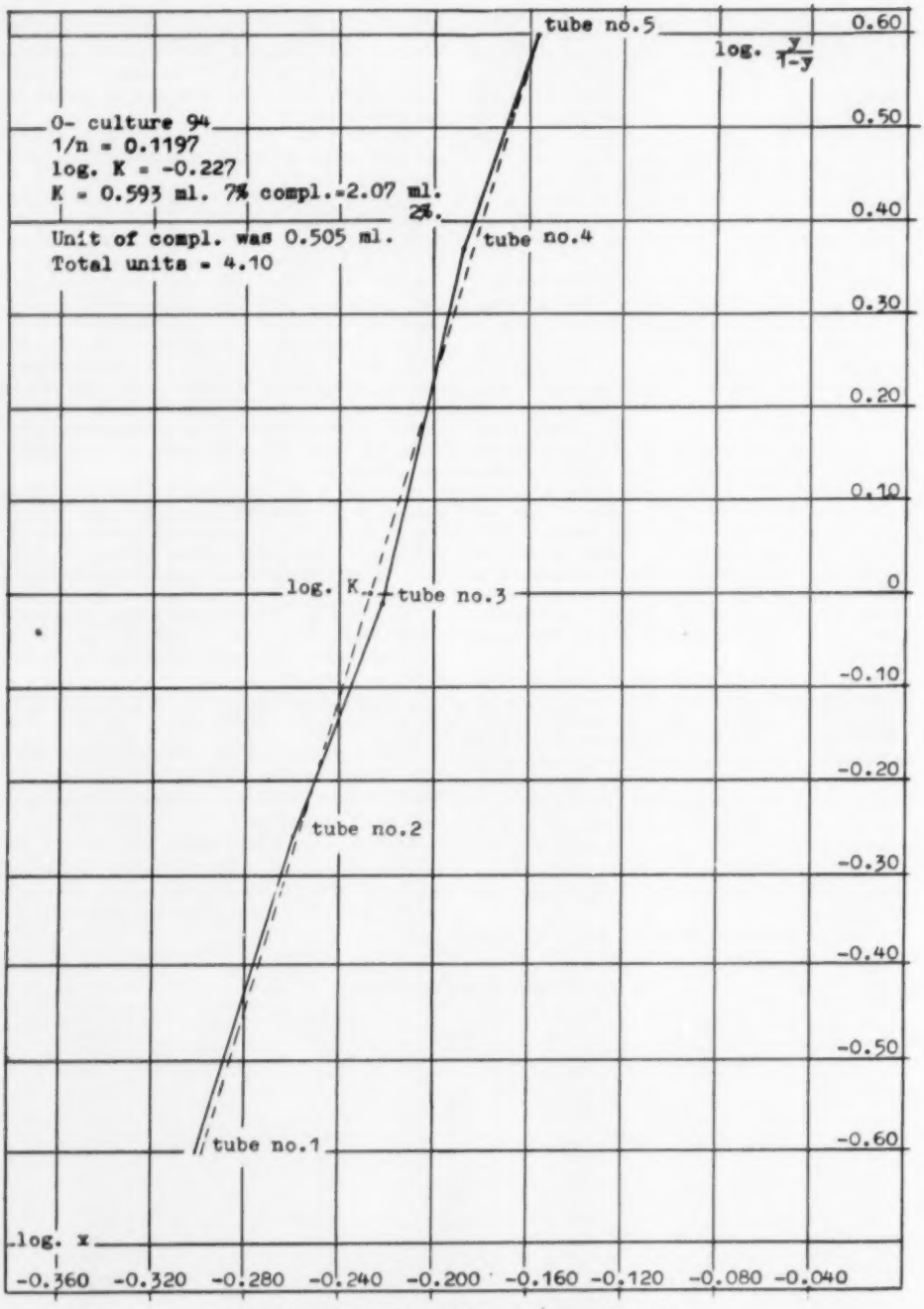
The antigen may be titrated after having estimated the unit of complement in the presence of the antigen and normal, inactivated guinea pig serum (table 4).

The optimal amboceptor and serum dilutions are used. Titrations of previous cultures give us an indication of the concentration of the complement necessary to use.

TABLE 3—Serum Titration

Tube No.	Physiol. salt solution (ml.)	Serum dilution (ml.)	Antigen (ml.)	Complement dilution (ml.)	Units	Hemolysis system (ml.)
1	1.25	0.40	0.50	0.25	2.5	1.60
2	1.20	0.40	0.50	0.30	3.0	1.60
3	1.15	0.40	0.50	0.35	3.5	1.60
4	1.10	0.40	0.50	0.40	4.0	1.60
5	1.05	0.40	0.50	0.45	4.5	1.60
6	1.00	0.40	0.50	0.50	5.0	1.60
7	0.95	0.40	0.50	0.55	5.5	1.60
8	0.90	0.40	0.50	0.60	6.0	1.60
9	0.85	0.40	0.50	0.65	6.5	1.60
10	0.80	0.40	0.50	0.70	7.0	1.60
11	0.75	0.40	0.50	0.75	7.5	1.60
12	0.70	0.40	0.50	0.80	8.0	1.60
13	0.65	0.40	0.50	0.85	8.5	1.60
14	0.60	0.40	0.50	0.90	9.0	1.60
15	0.00	0.40	0.50	1.50	15.0	1.60

It is necessary in the titration of the serum to estimate the unit of complement in the presence of the antigen and normal guinea pig serum. This has been done in order to eliminate the influence of the culture medium and the thermostable factors in the serum. We assume that the amount of these factors is the same in the normal inactivated serum as in the hyperimmune serum.



Graph 4—The titration of type O serum, culture 94.

TABLE 4—Example for Estimating the Unit of Complement in the Presence of Antigen and Inactivated Normal Guinea Pig Serum

Saline (ml.)	Complement 1 : 50 (ml.)	Normal guinea pig serum (ml.)	Culture antigen A 286 (ml.)	Hemolysis system (ml.)	Extinction	Hemolysis (%)	K quantity for 50 % hemolysis (ml.)
1.20	0.30	0.40	0.50	1.60	31.50	25.50	0.3748
1.15	0.35	0.40	0.50	1.60	43.00	40.00	0.3807
1.10	0.40	0.40	0.50	1.60	55.00	57.75	0.3750
1.05	0.45	0.40	0.50	1.60	62.00	69.25	0.3803
1.00	0.50	0.40	0.50	1.60	65.00	74.25	0.4014
0.95	0.55	0.40	0.50	1.60	69.50	.....	.....
0.90	0.60	0.40	0.50	1.60	76.00	.....	1.9122
0.85	0.65	0.40	0.50	1.60	78.50	.....	
.....	1.50	0.40	0.50	1.60	80.00	(after adjusting the galvanometer)	

In this titration the amount of complement which has been used is 1.9122 ml. for 5 tubes. The average K for 1 tube is $1.9122 \div 5 = 0.3824$ ml.

After incubation and centrifugation, the hemolysis can be estimated. An example of antigen titration is shown (table 5). The apparatus for the determination for hemolysis is first adjusted against tube 12 to compensate for the color present in the serum and antigen.

It must be noted that both types of titration have always been carried out with cultures harvested at or nearly at the time we believed the cultures to be at the peak of growth (20 hours). These are indications that titrations done after longer or shorter incubation periods give a more variable correlation.

The values of 1/n and K can be calculated using the von Krogh formula and the method of least squares.

The values of log x are plotted on the abscissa against the corresponding values of log y on the ordinate. An almost straight line can be drawn through the co-ordinating points (the solid line in graph 4).

In the example given, the value of the unit of complement is 0.505 ml. of a 2 per cent solution; of 1/n 0.1197; for log K −0.227. The concentration of complement used was 7 per cent.

If $\log K = -0.227$ then K is $\frac{0.593 \cdot 3.5}{0.505} = 4.1$ units.

The value of log K for tube 1 is, accordingly, the relation $\log x = \log K + 1/n \cdot \log \frac{y}{1+y}$, substituting for x 0.50, for 7, 20, and for 1/n 0.1197; −0.299. In the same way the value of log K can be calculated for tube 5 being −0.155. Between these two points a straight line (the broken line in graph 4) can be drawn.

The calculated correlation coefficient (degree of accuracy) between these two lines is 99.5 per cent.

The von Krogh formula can be written in another manner substituting for $\log x = p$; for $\log \frac{y}{1-y} = q$; for $K = a$ and for $1/n = b$, namely as: $p = a + bq$. This formula expresses the function (position) of a straight line.

The proximity of the broken and the solid lines shows that the use of the von Krogh formula for the complex antigen,

TABLE 5—Antigen Titration of Type O Culture No. 94

Tube No.	Physiol. salt solution (ml.)	Complement 7 % (ml.)	O Antiserum 5 % (ml.)	Culture antigen (ml.)	Hemolysis system (ml.)	Extinction	Hemolysis (%)	K quantity for 50 % hemolysis (ml.)
1	1.20	0.30	0.40	0.50	1.60	5.00	.....	.....
2	1.15	0.35	0.40	0.50	1.60	8.50	.....	.....
3	1.10	0.40	0.40	0.50	1.60	10.50	.....	.....
4	1.05	0.45	0.40	0.50	1.60	17.75	.....	.....
5	1.00	0.50	0.40	0.50	1.60	26.50	20.00	.....
6	0.95	0.55	0.40	0.50	1.60	39.50	35.50	.....
7	0.90	0.60	0.40	0.50	1.60	49.50	49.25	0.593
8	0.85	0.65	0.40	0.50	1.60	62.50	70.00	.....
9	0.80	0.70	0.40	0.50	1.60	68.25	80.00	.....
10	0.75	0.75	0.40	0.50	1.60	73.00	.....	.....
11	0.70	0.80	0.40	0.50	1.60	76.00	.....	.....
12	.....	1.50	0.40	0.50	1.60	80.00	(after adjusting the galvanometer)	

serum, complement, and hemolytic system is admissible.

Fifty-two type O cultures, 56 type A cultures, and 73 type C cultures were titrated by this method. The results of these cultures obtained by the complement-fixation method were compared with the titration results of these cultures in susceptible cattle using the method of Henderson.[10] The results of the comparative tests show a definite correlation between the complement fixing and the infectivity property of the virus. The fixation of one unit of complement by foot-and-mouth disease culture virus was found to correspond with 128,000 i.d.$_{50}$ for type A cultures; 100,000 i.d.$_{50}$ for type C cultures; and 125,000 i.d.$_{50}$ for type O cultures.

Immunogenic Properties of Vaccine Prepared from Cultures Evaluated by Titration in Cattle and by Complement Fixation.—From 22.5 liters of type C culture No. 94, 45 liters of vaccine were prepared containing 2.5 per cent virus, and from 45 liters of the same culture, 405 liters containing 5 per cent virus were prepared. Both cultures fixed 7.1 units of complement. The vaccines have been prepared following the method of Waldmann *et al.*[14] with the basic difference that tissue-cultured virus is used instead of cattle virus. The vaccines were found to be free from contamination and the innocuity test was negative.

Fifteen milliliters of each vaccine was given subcutaneously in the dewlap of each of 4 susceptible cattle. Three months after vaccination 3 unvaccinated controls were put with the vaccinated animals. One of the controls was injected intradermally in the tongue with a 2 per cent virus suspension of type C virus. Mouth and tongues of the vaccinated animals and the 2 other controls were swabbed with a towel moistened with the same suspension.

The 3 controls showed fever on the second and third day. On the fourth day the vaccinated animals were injected with a 10 per cent virus suspension in the tongue. All the animals were slaughtered 12 days after signs of illness had first appeared. Postmortem examination of the control animals showed a reaction on the surface of the tongue, the palate, and the four feet. Except for reaction at the site of inoculation, no lesions were found in any of the vaccinated animals.

SUMMARY

1) A definite correlation between the complement-fixation titration and the titration method employing susceptible cattle was found following the titrations of 181 cultures of foot-and-mouth disease virus.

2) That amount of foot-and-mouth disease culture virus required to fix one unit of complement was found to correspond with 128,000 infectious dose (i.d.)$_{50}$ of type A virus; with 100,000 i.d.$_{50}$ of type C virus; and with 125,000 i.d.$_{50}$ of type O virus.

3) Animals injected subcutaneously with 15 ml. of vaccine, which had been evaluated by the complement-fixation method, were refractive upon challenge three months after vaccination.

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Sulfaquinoxaline and Sulfamerazine in the Treatment of Experimental Infections with *Eimeria Bovis* in Calves

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SULFAQUINOXALINE has been widely used for the prevention and treatment of coccidiosis in poultry.

It was observed by Dunlap [1] to be effective for treatment of coccidiosis due to *Eimeria zurnii* in calves and was used as an enema in the treatment of coccidiosis in older animals, either with or without concurrent oral treatment with sulfamerazine.[2] Davis and Bowman [3] found that treatment with sulfaquinoxaline reduced the severity of experimental infections with Coccidia but that sulfaquinoxaline gave less protection against experimental coccidiosis in calves than did sulfamethazine.[4] It is listed by Foster [5] as a therapeutic agent considered to be in the experimental stage for coccidiosis in cattle.

Sulfamerazine, which is similar to sulfamethiazine in characteristics relating to absorption and duration of concentrations in the blood,[6] might also be expected to give good results in protecting calves against coccidiosis. The purpose of this study was to determine whether medication with sulfaquinoxaline or with sulfamerazine at particular times in relation to inoculation would result in alteration of experimental infections with *Eimeria bovis*.

MATERIALS AND METHODS

Four experiments involving 12 calves each were conducted, one with sulfaquinoxaline, two with sulfamerazine, and one with sulfaquinoxaline and sulfamerazine. The experiments were conducted successively from April, 1954, to August, 1955. The calves, Holstein-Friesian males obtained usually at an age of 1 to 3 days, were housed in outdoor individual pens similar to those described by Davis.[7] The pens were either moved or thoroughly cleaned and provided with fresh straw at weekly intervals. The calves were fed raw whole milk until 2 to 3 weeks of age when this was gradually replaced by a mixture made from skim milk powder. After the first few weeks, alfalfa hay and oats were fed to supplement the milk. Daily collection of fecal samples was begun when the calves were between 2 and 3 weeks old, and a technique similar to that of Boughton [8] was used for examination for oocysts.

When the calves were 8 to 25 days old they were given a mixture of sporulated oocysts obtained from fecal samples collected from calves in previous experiments. Each inoculum consisted of *Eimeria bovis*, 90 per cent or more, with several other species of Coccidia, including *Eimeria auburnensis*, *E. zurnii*, and *Eimeria ellipsoidalis*. The oocysts were given by the nippled flask method, shown in previous work to be about as effective as introduction into the abomasum or use of capsules.[9] An estimated 0.5 to 1.0 million oocysts were given to the calves in one or more doses in the various experiments. Experiments were continued four to six weeks after inoculation.

In the experiment with sulfaquinoxaline, each of the 12 calves received an estimated million oocysts; 6 received a single dose and the other 6 received five equal doses on alternate days. Three of the calves in each inoculation group received no treatment; the other 3 were given two series of treatments with sulfaquinoxaline, beginning on the day of inoculation and on the twenty-first day after inoculation. In each series, the drug was given at the rate of 1.0 gr. per pound of body weight for one day and 0.5 gr. per pound for three days. The drug was administered in capsules. The groups of calves were balanced as nearly as possible with respect to age and weight.

The calves were weighed at the time of inoculation and at the end of the experiment. Observations of temperatures were taken daily, beginning about a week after inoculation. Blood samples were taken from all of the calves at weekly or semi-weekly intervals beginning at the time of inoculation. Total erythrocyte, total leukocyte, and differential leukocyte counts were made, as well as determination of hemoglobin. Calves dying during the experiment were examined as soon as possible after death. The colon, cecum, and lower part of the small intestine were examined for evidences of injury due to coccidiosis; scrapings were made and tissues fixed. Other organs were also examined and in the later part of the experiment, when indications of possible toxicity of the drug were noticed, samples of kidney tissue were fixed.

In the two experiments with sulfamerazine and in the experiment with sulfamerazine and sulfa-

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quinoxaline, the methods used were similar to those of the experiment with sulfaquinoxaline, except that the calves were separated into three groups of 4, and each calf received an estimated 0.5 million oocysts in two equal doses on successive days. In the first of these experiments, one group of calves was given a treatment beginning on the day before inoculation and a second treatment beginning 13 days after inoculation. A second group was given a treatment beginning 13 days after inoculation. In the second experiment, one group of calves was given a treatment beginning 13 days after inoculation and a second group was given a treatment beginning 18 days after inoculation. In the third experiment, one group of calves was given a treatment of sulfamerazine beginning 13 days after inoculation, while a second group was given a similar treatment with sulfaquinoxaline. In each experiment, one group of 4 calves received no treatment. Each treatment consisted of the administration by capsule of 1.0 gr. of drug per pound of body weight for two days and 0.5 gr. for two days. In the last two of these experiments, the only blood determinations that were made were total leukocytes. Calves were weighed at weekly intervals.

RESULTS

In the experiment with sulfaquinoxaline (table 1), all the calves that received a single dose of oocysts experienced bloody diarrhea and 5 of the 6 calves, including 3 treated and 2 untreated, died from the twentieth to the twenty-fourth day after inoculation, evidently as a result of coccidiosis. The treated calves died earlier (day 20 to 22) than the untreated (day 23 to 24). The only surviving calf was untreated. Apparently, treatment beginning on the day of inoculation was not effective in preventing development of coccidiosis. There was no apparent difference in discharge of oocysts in treated and untreated calves.

In the 6 calves given five doses of oocysts, there were severe signs of coccidiosis in 2 calves, 1 untreated (587) and 1 treated (590); signs of moderate degree in 2 calves, 1 untreated (584) and 1 treated (591); and signs of slight degree in 2 calves, 1 untreated (588) and 1 treated (592). The 2 calves with severe coccidiosis died during the experiment, the untreated calf on the twenty-first day after first inoculation and the treated calf on the twenty-seventh day. The calves with a moderate degree of coccidiosis showed no gain in weight during the 32 days of the experiment and both died about two weeks later. The untreated calf with a slight degree of coccidiosis made a gain in weight of 38 per cent, while the treated calf made a corresponding gain of eight per cent. In this untreated calf (588), there was a discharge of moderate numbers of oocysts of *E. bovis* and other species, beginning a few days prior to inoculation and lasting about two weeks. The natural infection, indicated by this discharge of oocysts, probably resulted in an immunity which was associated with the relative mildness of the experimental infection and the large gain in weight. In this group of 6 calves, the discharge of oocysts in the treated and untreated calves was similar, except for 1 treated calf (592) that discharged relatively few oocysts. This group also showed little or no beneficial effect of the sulfaquinoxaline in preventing the development or alleviating the signs of coccidiosis. The only indications of influence of the treatment on the coccidiosis were in calf 592, in which the oocyst discharge was relatively light, and in calf 590 in which death occurred six days later than in the corresponding untreated calf. It should be noted that the calves in this group received two series of treatments, while those in the group of calves given a single inoculum died before or about the time of the beginning of the second series of treatments.

In the calves that died, apparently as a result of coccidiosis, examination of sections of the colon and cecum confirmed the diagnosis. Bacteriological examinations of the colon at necropsy of these calves revealed no pathogenic organisms. Results of the blood examinations showed nothing of significance.

In the first experiment with sulfamerazine (table 2), calves that received two treatments, one beginning on the day before inoculation and the other beginning 13 days after inoculation, showed no definite signs of coccidiosis. However, 3 of the 4 calves had a brief diarrhea at the time signs of coccidiosis usually appear. Except for a few oocysts of species other than *E. bovis*, no oocysts were detected in any of the 4 calves until 29 days after inoculation, about ten days later than oocysts usually appear in experimental infections with *E. bovis*. Concentrations of oocysts were relatively low. Three of the calves made good gains in weight; 1 calf became moribund, evidently as a result of complications originating from a navel infection.

TABLE 1—Results of Use of Sulfaquinoxaline in Calves Experimentally Infected with Eimeria Bovis with Treatment Beginning at the Time of Inoculation and on the Twenty-first Day Thereafter

Calf (No.)	Age-weight of calf at inoculation (days-lb.)	Inoculation* (doses)	Treatment**	Occurrence of diarrhea and tenesmus: Type	Occurrence of diarrhea and tenesmus: Time (days after inoc.)	Discharge of *E. bovis* oocysts: Peak (thousands-† day after inoc.)	Discharge of *E. bovis* oocysts: Time (days after inoc.)	Percentage wt. gain in 32 days	Remarks
584	17–84	5	None	Bloody diarrhea Diarrhea	19 20–25	4.5–22	12–24 26–30	0	Moribund, killed 46 days after inoc.; no definite abnormalities found.
587	22–80	5	None	Diarrhea Bloody diarrhea Tenesmus	17–18 19–20 19–20	20.0–19	18–20	....	Died 21 days after inoc.; death evidently due to coccidiosis.
588	20–87	5	None	Slight diarrhea	17–24	3.5–20	19–25	38	Natural infection with *E. bovis* and other species; discharge of oocysts from 1–14 days after inoc.
590	13–76	5	Treated	Diarrhea Bloody diarrhea Tenesmus	18,25–26 20–24 26	6.5–21	16–26	....	Died 27 days after inoc.; coccidosis probably contributed as cause of death.
591	13–74	5	Treated	Diarrhea	18–26	10.0–21	18–28	0	Died 41 days after inoc.; no definite abnormalities found.
592	22–77	5	Treated	Diarrhea	23–24	0.4–22	19–24	8	.....
585	10–88	1	None	Diarrhea	13–14,19 22, 25				.....
				Bloody diarrhea	20–21	12.0–21	19–29	−5	
594	24–90	1	None	Diarrhea Bloody diarrhea Tenesmus	17 18–22 21–22	15.0–19	18–21	....	Natural infection with *E. bovis* discharge of oocysts 6, 9–10 and 12 days after inoc.
586	25–90	1	None	Bloody diarrhea	18–22	50.0–20	14–22	....	Died 24 days after inoc.; death evidently due to coccidiosis.
589	15–78	1	Treated	Diarrhea Bloody diarrhea	13, 17 18–19	0.6–19	18–19	....	Died 20 days after inoc.; death evidently due to coccidiosis.
595	15–79	1	Treated	Diarrhea Bloody diarrhea Tenesmus	16 17–21 21	20.0–21	18–21	....	Died 22 days after inoc.; death evidently due to coccidiosis.
593	24–69	1	Treated	Bloody diarrhea Tenesmus	19–21 20–21	20.0–20	15,18–21	....	Died 22 days after inoc.; death evidently due to coccidiosis.

* Inoc. consisted of approximately 1,000,000 oocysts, chiefly *E. bovis*, given in a single dose or in 5 equal doses on alternate days.

** Treatment was at the rate of 1.0 gr. per pound of body wt. for one day and 0.5 gr. for three days.

† Numbers of oocysts refer to counted or estimated number in a drop removed by a loop after flotation from about 0.1 tablespoon of feces.

In the groups of 4 calves which were treated beginning 13 days after inoculation, there were no signs of coccidiosis other than a slight diarrhea which was present for a few days. Except for a few oocysts of species other than *E. bovis* in some of the calves, no oocysts were seen at the time these usually appear in experimental infections with *E. bovis*. Oocysts were found in relatively small numbers in 3 of the 4 calves beginning about ten days later than the time of usual appearance. Gains in weight were comparable to those of the first group. One calf died, evidently as a result of bloat.

In the second experiment with sulfamerazine (table 3), the group of 4 calves that was given treatment beginning 13 days after inoculation gave similar results to those of the corresponding group in the first experiment. However, 1 calf experienced tenesmus for two days. In 3 of the calves, small numbers of oocysts were discharged at a time corresponding to the first few days of the normal period of oocyst discharge in experimental *E. bovis* infections. There was then a negative period for several days, followed by a period of discharge of larger numbers of oocysts. The second period corresponded in time with the discharge found in the first experiment.

In the group of 4 calves which received treatment beginning 18 days after inoculation, there was no distinct difference in results as compared with those of the untreated calves. The only calf in which there was evidence of benefit as a result of treatment was 1 (502) in which the symptoms of coccidiosis and the period of oocyst discharge were of relatively short duration and the gain in weight relatively

TABLE 2—Results of Use of Sulfamerazine in Calves Experimentally Infected with Eimeria Bovis with Treatment Beginning at the Time of Inoculation and Repeated 13 Days Later or Beginning 13 Days After Inoculation

Calf (No.)	Age-weight of calf at inoculation (days-lb.)	Treatment (series)	Occurrence of diarrhea and tenesmus: Type	Occurrence of diarrhea and tenesmus: Time (days after inoc.)	Discharge of *E. bovis* oocysts: Peak (thousands-† day after inoc.)	Discharge of *E. bovis* oocysts: Time (days after inoc.)	Percentage wt. gain in 43 days	Remarks
302	18–107	2*	Diarrhea	20	0.07–31	27–36	46	
303	10–94	2	Slight diarrhea Diarrhea	19,23 20–22	0.01–29	29,31		Became moribund and was killed 39 days after inoc.; evidently had uremia and infectious cystitis, probably arising from navel infection.
304	14–73	2			0.01–29	27–31	47	.....
307	12–102	2	Diarrhea	19	0.2 –31	29–40	23	.....
301	16–95	1*	Diarrhea	18–20	0.01–38	38	27	Probable natural infection with *E. bovis* and other species; discharge of oocysts 9–15 days after inoc.
305	14–95	1	Slight diarrhea	19,22,24	0.04–29	27–36	40	.....
308	14–104	1	Slight diarrhea Diarrhea	17–18,21 19–20	0.01–29	29	50	Probable natural infection with *E. bovis* and other species; discharge of oocysts 11–16 days after inoc.
311	11–95	1	Slight diarrhea	22	0.2 –33	27–33	....	Died 36 days after inoc. evidently as a result of bloat.
306	15–96	....	Diarrhea Bloody diarrhea	19–21,24–27 22–23	0.6 –22	17–28,30 36	....	Died 39 days after inoc.; acute infection of upper respiratory tract caused by hemolytic streptococcus.
309	16–82	....	Slight diarrhea Diarrhea	21,28,31 22–27,30	3.0 –21	12–32	....	Early discharge of oocysts probably due to natural infection.
310	13–105	....	Diarrhea Bloody diarrhea Tenesmus	17–19,25–29 31–33 20–24,30 23–27	0.4 –22	18–31	....	Died 34 days after inoculation evidently as a result of coccidiosis.
312	10–102	....	Diarrhea Bloody diarrhea	17,19 20–25	4.0 –21	18–25	....	Became moribund, was killed 26 days after inoc.; moribund condition evidently caused by coccidiosis.

* Each series was at the rate of 1.0 gr. per pound of body weight for two days and 0.5 gr. per pound for two days.

† Numbers of oocysts refer to counted or estimated number in a drop removed by a loop after flotation from about 0.1 tablespoon of feces.

TABLE 3—Results of Use of Sulfamerazine in Calves Experimentally Infected with Eimeria Bovis, with Treatment Beginning 13 or 18 Days After Inoculation

Calf (No.)	Age—weight of calf at inoculation (days–lb.)	Treatment, interval after inoc.	Occurrence of diarrhea and tenesmus		Discharge of *E. bovis* oocysts		Percentage wt. gain in 41 days	Remarks
			Type	Time (days after inoc.)	Peak (thousands†–day after inoc.)	Time (days after inoc.)		
501	22–114	1 series*	Diarrhea	18–21,24	0.01–20	20–22,28–29,31,33	30	.....
		13 days	Tenesmus	20–21				
505	18–95	1 series	Slight diarrhea	18,20	1.0 –28	18,26–30	40	Probable natural infection with *E. bovis* and other species; discharge of oocysts 8–15 days after inoc.
		13 days	Diarrhea	19				
507	17–82	1 series	.....	.....	3.4 –27	20,26–31 33–34	50	Probable natural infection with *E. bovis* and other species; discharge of oocysts 12–17 days after inoc.
		13 days						
512	13–58	1 series	Slight diarrhea	19–20	0.8 –28	18,21 27–30	78	.....
		13 days						
502	22–87	1 series*	Diarrhea	19–25	3.0 –19	18–19, 22–23	41	.....
		18 days	Tenesmus	20–24				
504	19–97	1 series	Diarrhea	16,18–28	4.0 –19	18–25,27–28,31	21	.....
		18 days	Tenesmus	23–24				
509	17–91	1 series	Diarrhea	16–18	20.0 –19	15–26		Died 27 days after inoc., evidently as a result of coccidiosis; early discharge of oocysts probably due to natural infection.
		18 days	Bloody diarrhea	19–26				
			Tenesmus	20–26				
510	15–91	1 series	Diarrhea	18	7.0 –19	18–31	6	Died during week after experiment was concluded.
		18 days	Bloody diarrhea	19–37				
			Tenesmus	20–22				
503	19–104	.....	Diarrhea	18	5.4 –16	15–22		Died 23 days after inoc.; autopsy showed severe damage due to coccidiosis, multiple liver abscesses, perforated abdomasum; early discharge of oocysts probably due to natural infection.
			Bloody diarrhea	19–22				
			Tenesmus	20–22				
506	17–82	.....	Diarrhea	18–19,21–26,31–34	7.0 –20	13–29	29	Early discharge of oocysts probably due to natural infection.
			Bloody diarrhea	20				
			Tenesmus	20–26,31				
508	15–80	.....	Bloody diarrhea	18–25	4.2 –19	15–25		Died 26 days after inoc., evidently as a result of coccidiosis; early discharge of oocysts probably due to natural infection.
			Tenesmus	20–25				
511	13–86	.....	Diarrhea	19–36	19.0 –21	19–25 31–32,34	24	.....
			Tenesmus	21–36				

* Each series was at the rate of 1.0 gr. per pound of body weight for two days and 0.5 gr. per pound for two days.

† Numbers of oocysts refer to counted or estimated number in a drop removed by a loop after flotation from about 0.1 tablespoon of feces.

great. One of the 8 treated calves died during the experiment as compared with 2 of the 4 untreated calves, but an additional treated calf died during the week following conclusion of the experiment. Therefore, it appears that treatment timed at this stage of the disease is of doubtful value.

In the experiment with sulfamerazine and sulfaquinoxaline (table 4), the untreated calves experienced less severe coccidiosis than in the other two experiments. Bloody diarrhea was present in 3 of 4 of these calves, but there was no mortality and 3 of the 4 made good gains in weight. The 4 calves treated with sulfamerazine experienced only slight diarrhea for a few days and discharged relatively few oocysts. The late discharge of oocysts was less pronounced and consistent than in the other two experiments.

The 4 calves treated with sulfaquinoxaline showed a more severe diarrhea than those treated with sulfamerazine, but no apparent blood was discharged, although 1 calf showed a brief discharge of tissue. Discharge of oocysts from most of these calves was at higher levels than from calves treated with sulfamerazine, but distinctly less than from untreated calves. The peak in concentration of oocysts occurred later than in untreated calves. Sulfaquinoxaline appeared to be beneficial, although to a somewhat lesser extent than sulfamerazine when given at this stage of the disease. Since there was only one experiment involving a comparison of the efficacy of sulfaquinoxaline and sulfamerazine, further work must be done before a valid conclusion as to the relative value of these drugs can be reached.

Several of the calves in each of these three experiments showed natural infections, with discharges of oocysts occurring not earlier than ten days prior to the normal period of discharge of the experimental infection. The degree to which these natural infections influenced the experimental infections is unknown, but in most cases the effect was probably not of much significance.

The total leukocyte counts showed no evidence of toxicity of the drugs used in any of these experiments. In the second experiment, the 3 calves which died as a result of coccidiosis or coccidiosis in combination with other causes (calf 503) showed a marked increase in leukocyte count a day or two before death.

DISCUSSION

The only other controlled experiments involving use of sulfaquinoxaline for coccidiosis have been conducted by Davis and Bowman. They found that calves treated with sulfaquinoxaline were less severely affected and gained more weight than did untreated ones.[3] In later work,[4] they conducted two tests involving 16 calves in which the effects of sulfaquinoxaline and sulfamethazine were compared. In both tests, weight gains were less in calves receiving sulfaquinoxaline than in untreated calves or in those receiving sulfamethazine, although the difference was slight between the first two groups in one test. There was no appreciable difference in amount of diarrhea in the calves given sulfaquinoxaline as compared with untreated calves. Discharge of oocysts was lighter in calves given sulfaquinoxaline than in untreated calves but heavier than in those given sulfamethazine. In one test, 2 of the untreated calves died of coccidiosis. In the present study, in which timing of treatment and spacing of inoculation differed from that of Davis and Bowman, no consistent effect of sulfaquinoxaline in preventing or alleviating coccidiosis or in decreasing the discharge of oocysts was found in calves treated at the time of inoculation with a second treatment 21 days later, but in calves given a treatment beginning on the thirteenth day after inoculation there was some protection against coccidiosis, although less than resulted from sulfamerazine.

Boughton [8] obtained favorable results with sulfaguanidine in the case of experimental infections with *E. bovis* when the drug was given daily for 21 days beginning two days after inoculation or for eight days beginning 13 days after inoculation. Unfavorable results were obtained when the drug was given for eight days beginning three days before or five days after inoculation. Boughton speculated that the drug might act against the merozoites during the time they are exposed to the contents of the intestinal lumen. It was shown in later work [10] that the time at which the administration of the drug begins to be effective corresponds with the approximate time merozoites are released

TABLE 4—Results of Use of Sulfamerazine as Compared with Sulfaquinoxaline in Calves Experimentally Infected with Eimeria Bovis, with Treatment Beginning 13 Days After Inoculation

Calf (No.)	Age–weight of calf at inoculation (days–lb.)	Treatment*	Occurrence of diarrhea and tenesmus		Discharge of *E. bovis* oocysts		Percentage wt. gain in 28 days	Remarks
			Type	Time (days after inoc.)	Peak (thousands†–day after inoc.)	Time (days after inoc.)		
551	8–93	Sulfamerazine	Slight diarrhea	22–24	0.01 –21,23	21–23	27	.....
552	8–75	Sulfamerazine	Slight diarrhea	22–23,25	0.001–20,23	20,23	36	.....
553	13–94	Sulfamerazine	Slight diarrhea	19,22–25	0.04 –31	19–23, 30–31	22	.....
554	12–98	Sulfamerazine	Slight diarrhea	24,28	1.6 –15	15–21,23–24,26–30	24	Early discharge of oocysts may have been due to natural infection.
555	8–98	Sulfaquinoxaline	Slight diarrhea Diarrhea	23–25,26 25	1.8 –25	21–30	28	Traces of tissue and blood appeared 26 and 28 days after inoculation.
556	13–110	Sulfaquinoxaline	Slight diarrhea Diarrhea	17,22,23–24 19	0.86 –27	17,19–21, 23,26–29	34	Probable natural infection with *E. bovis* and other species; discharge of oocysts 14–15 days after inoc.
557	12–97	Sulfaquinoxaline	Slight diarrhea Diarrhea Tissue	23,27,30 26,28–29 27	1.3 –27	18–19,23, 26–30	16	Probable natural infection with *E. bovis* and other species; discharge of oocysts 12–16 days after inoc.
558	11–98	Sulfaquinoxaline	Slight diarrhea Diarrhea	19 29–31	0.86 –28	19–21,23, 26–31	21	.....
559	15–92	.....	Slight diarrhea Diarrhea	19–21,23–27 23	15.8 –20	12,16–31	30	Early discharge of oocysts probably due to natural infection.
560	11–73	.....	Slight diarrhea Diarrhea Bloody diarrhea	17,19,23, 25,27,29 20–22 24	10.7 –23	19–26,28–29,31	30	.....
561	12–94	.....	Slight diarrhea Diarrhea Bloody diarrhea Tenesmus	18–19 20 21–26,29 24	6.6 –20	19–28	30	.....
562	8–107	.....	Slight diarrhea Bloody diarrhea	18–21,27–29 22–26	10.7 –23	16–26,31	10	Early discharge of oocysts may have been due to natural infection.

* Each series was at the rate of 1.0 gr. per pound of body weight for two days and 0.5 gr. per pound for two days.

† Numbers of oocysts refer to counted or estimated number in a drop removed by a loop after flotation from about 0.1 tablespoon of feces.

from the schizonts. The present work agrees with that of Boughton in the finding that during the period beginning thirteen days after inoculation *E. bovis* is vulnerable to action of drugs. In the present work, however, the drug was given for four days only, while in Boughton's work it was given for eight. It was also found in the present work that treatment beginning 18 days after inoculation is relatively ineffective. It is likely that by this time the sexual stages have developed sufficiently so that irreversible damage to the tissues has already occurred. Thus the period during which *E. bovis* is vulnerable to action of the drug includes the time from 13 to 17 days after inoculation. With respect to the earlier stages of the disease, the present findings also agree with those of Boughton in indicating that *E. bovis* is relatively invulnerable immediately following inoculation. Sulfaquinoxaline was found to be ineffective at this time, while it was effective to some degree when given beginning 13 days after inoculation.

Further work is needed on the timing of treatment in relation to the stage of the disease and on the relative efficacy of sulfamerazine, sulfamethazine, and other drugs.

SUMMARY

In four experiments, each with 12 calves, all were inoculated with 0.5 to 1.0 million oocysts, chiefly *Eimeria bovis*, in one to five doses. In each experiment, one portion of the calves was untreated and the remaining portion was treated.

In one experiment, sulfaquinoxaline was given at the rate of 1.0 gr. per pound of body weight for one day and 0.5 gr. for three days. Treatment was begun at the time of first inoculation and 21 days later. There was little or no protection against coccidiosis.

In three experiments, sulfamerazine or sulfaquinoxaline was given at the rate of 1.0 gr. per pound of body weight for two days and 0.5 gr. for two days. In one experiment, treatment with sulfamerazine was begun one day before inoculation and repeated 13 days after inoculation in one group, and in another group, a single treatment with sulfamerazine was given beginning 13 days after inoculation. In a second experiment, treatment with sulfamerazine was begun 13 days after inoculation in one group and 18 days in another. In a third experiment, sulfamerazine was given to one group and sulfaquinoxaline to another beginning 13 days after inoculation. All groups treated beginning 13 days after inoculation showed light or no signs of coccidiosis and discharged few oocysts; sulfaquinoxaline was somewhat less effective than sulfamerazine. Treatment beginning 18 days after inoculation was ineffective. The stages of *E. bovis* which occur 13 to 17 days after inoculation apparently are most susceptible to these drugs.

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Experimental Leptospirosis in Pregnant Swine

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LEPTOSPIROSIS is being recognized more frequently today and it is assuming a place of importance among swine diseases. Bryan *et al.*[1] and Bohl *et al.*[2] reported outbreaks of the disease in herds of swine in which a large proportion of the pigs farrowed during one season were born dead or died soon after birth. *Leptospira pomona* was isolated from animals in these herds, and it was presumed to be the cause of the heavy losses. Additional outbreaks in herds of swine in this area are described in another paper.[3]

The objectives of the experiment described in this report were to obtain some information on the methods of transmission of *L. pomona*, to determine the role of *L. pomona* in abortion in pregnant sows, and to determine the stages of pregnancy at which the sows are most susceptible to reproductive failure.

MATERIALS AND METHODS

The crossbred swine used in this experiment were from the experiment station herd and were approximately 1 year old. The 16 gilts were maintained in small pens in an isolated building, 2 in each pen, until about one month prior to expected farrowing when they were placed in individual pens. They were fed a ration of ground feed containing approximately 15 per cent protein and with no added antibiotics.

Exposure to *L. pomona* was accomplished by two methods. In 6 of the gilts, 1.5 ml. of culture was deposited at the cervical opening by means of an insemination tube and syringe within one to 24 hours following breeding. Nine other gilts were exposed at different stages of pregnancy by dropping approximately 1 ml. of culture onto the conjunctiva of one eye and the same amount of culture in the nasal cavity. Three were exposed at approximately one month, 3 at about two months, and 3 at approximately three months after breeding. One gilt was maintained as an uninoculated control.

The strain of *L. pomona* used for experimental infection was originally isolated from the blood of a weak pig within six hours of birth. The strain was maintained in Schüffner's medium for a few months. A culture of this strain was used to expose 4 of the 6 gilts intravaginally; the other 2 were infected with a blood culture from gilt 394, previously exposed by the vaginal route. Prior to its use for infecting the 9 gilts conjunctivally and nasally, the original strain was passed serially in 3, 60-lb. pigs. A blood culture from the third pig in Schüffner's medium was the source of inoculum.

The Schüffner's medium was prepared with deionized water and the pH was adjusted to 7.0 to 7.1. Amounts of 0.05 to 0.5 ml. of blood, obtained from the anterior vena cava, were introduced into tubes of the medium. The contents were examined at intervals, usually weekly, by dark-field microscopy. Incubation of the cultures at 30 C. was continued for at least one month before recording them as negative.

Serological tests were performed with living cultures of *L. pomona* (strain NIH–1) which had grown in Schüffner's medium for three to seven days. The agglutination-lysis test, as previously described,[2] was used.

Examination of urine for the presence of Leptospira was accomplished in three ways with one or more of the procedures used on each specimen: (1) direct examination of the freshly collected urine was made by dark-field microscopy using a Spencer dark-field condenser with a strong source of reflected light, a 15X ocular and a 10X objective for examination of an open slide or a 40X objective (600 magnification) for coverslip preparations; (2) examination by the above procedure following centrifugation with a spinco high speed centrifuge at 10,000 r.p.m. for five minutes to concentrate the Leptospira without immobilizing them; and (3) injection of 0.5 to 2.0 ml. of the urine intraperitoneally into each of 1 or more hamsters. On the fourth day the hamsters were bled aseptically by cardiac puncture and 0.1 to 0.5 ml. of blood was placed in tubes of Schüffner's medium and examined at weekly intervals for 30 days for the presence of Leptospira.

Search for *L. pomona* in tissues of aborted or weak pigs and tissues of the gilts at slaughter was carried out by grinding liver or other organs in Schüffner's medium and 0.5 to 1.0 ml. of the tissue suspension was injected intraperitoneally into young hamsters. Blood samples were taken from the hamsters aseptically by cardiac puncture on the fourth day and the blood was cultured as described above.

RESULTS

Nine of the gilts had been bred when placed in the experiment. At the time of exposure, 3 gilts (152, 293, and 401) were 17 to 21 days pregnant; 3 gilts (155, 186,

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and 233) were 48 to 53 days pregnant; and 3 gilts (141, 396, and 421) were 71 to 73 days in pregnancy.

All of the 9 gilts, with the exception of 293, had a 1- or 2-day febrile response of 104 to 106 F. (table 1) and anorexia for one to three days starting the fifth to seventh day after the artificial infection.

This changed appetite, which varied from lack of interest in feed to complete anorexia, usually was evident either on the first day or, more frequently, on the second day of febrile response. Constipation, evidenced by scanty, dry, hard feces, was noted in most of the gilts.

Total leukocyte and differential counts made during this acute phase of the disease showed a great variation between animals, and from day to day in the same animal, with a trend toward an increased number of leukocytes during the fourth to the eighth day after exposure. This leukocytosis appeared to be an absolute increase in the number of neutrophils, many of which were immature forms.

Blood was obtained on alternate days during the acute phase of the disease for culture in Schüffner's medium and *L. pomona* was isolated from 7 of the 9 gilts; gilts 293 and 421 were negative. One of the 7 was positive on the second day and the remaining 6 on either or both the fourth and the sixth days after exposure (table 1).

Samples of blood were tested at intervals for the presence of antibodies for *L. pomona* in the agglutination-lysis test. Titers varying between 1:20 and 1:640 appeared on either the sixth or eighth day with the highest occurring on the tenth to the fourteenth days after infection. These "peak" titers ranged from 1:1280 to 1:81,920 (table 1).

Urine was collected from each of the gilts at irregular intervals for dark-field examination and inoculation of hamsters. *Leptospira pomona* was isolated by inoculation of hamsters with urine from gilt 233 on the twenty-first and twenty-fourth days and from gilt 141 on the twenty-first day after infection. Urine from the other gilts, examined from two to four times each by hamster inoculation, failed to reveal Leptospira.

The animals were watched carefully for any signs of abortion. No abnormalities were noticed in gilts 152, 293, and 401, which were exposed during the first month of pregnancy. Gilt 293 farrowed 2 pigs after 113 days of gestation. Both appeared normal but 1 was dead. The living pig was destroyed immediately but no leptospiras were demonstrated either in direct culture in Schüffner's medium or by hamster inoculation.

Gilt 152 farrowed 9 living, healthy pigs at 114 days. Some of the pigs had suckled when found and blood samples from these taken immediately contained antibodies for *L. pomona* in dilutions of from 1:160 to 1:640. Four of the pigs which were not permitted to nurse prior to collecting the blood sample possessed no demonstrable antibodies for Leptospira. One of these 4 pigs was destroyed but no *L. pomona* was found by direct culture or by hamster inoculation.

Gilt 401 farrowed 10 living pigs and 1

TABLE 1—Observations on 9 Pregnant Gilts Exposed to Leptospira Pomona by Conjunctival and Nasal Routes

Animal (No.)	Days pregnant at infection	First day of febrile response	Positive blood culture (days)	Antibody response: Day of first appearance	Antibody response: Day and level of highest titer**		Results of pregnancy
152	17	5*	4	6	14	20,480	Farrowed normally
293	21	....	....	6	12	81,920	Farrowed normally
401	17	5	2	8	12	5,120	Farrowed normally
155	53	5, 6	4, 6	6	10	5,120	Not pregnant, possibly early abortion
186	49	5	4	6	10	10,240	Not pregnant, possibly early abortion
233	48	6, 7	6	6	14	2,560	Aborted on day 86
141	71	6	6	8	10	1,280	In estrus day 79, rebred and normal pregnancy
396	71	6, 7	4	8	14	5,120	Farrowed normally
421	73	5, 6	....	8	14	5,120	Farrowed normally

* "Day" refers to the number of days following exposure to *L. pomona*.

** The titers are expressed as the reciprocal of the highest dilution of serum in which 50 per cent of the leptospiras were agglutinated or lysed.

dead pig. Two of the weaker pigs were destroyed but no leptospiras were found.

Of the 3 gilts which were in the second month of pregnancy when infected, all failed to farrow. Gilts 155 and 186 showed no signs of estrus or of abortion but, as the farrowing date approached, it became obvious that neither was pregnant. Gilt 155 developed a slight temperature elevation 39 days after exposure and during the subsequent week had a fair to poor appetite accompanied by constipation. These 2 gilts were slaughtered 86 days after infection and a kidney suspension from each was injected into hamsters but no leptospiras were demonstrated. The reproductive organs showed no direct evidence of resorption of fetuses; however, the uterine horns of gilt 186 were 40 inches long which would seem to be larger than expected in a normal, virgin sow.

Gilt 233 aborted 10 dead fetuses on gestation day 86, and 38 days after exposure. The fetuses weighed 1½ to 1¾ lb. each; 6 appeared normal but 1 was emaciated and underdeveloped, 1 was extremely edematous with excessive fluid in body cavities and throughout the tissues, 1 was slightly edematous with some fluid in the abdominal cavity, and 1 was badly decomposed. Dark-field examination, direct culture in Schüffner's medium, and hamster inoculation failed to reveal living leptospiras in the aborted fetuses.

Of the 3 gilts exposed during the third month of pregnancy, gilts 396 and 421 farrowed normally. Gilt 396 farrowed 4 large, healthy pigs at 115 days; 2 of these were destroyed at birth, but dark-field examination, direct culture, and hamster inoculation failed to reveal any Leptospira. Gilt 421 farrowed 10 pigs at 114 days; 6 of these were normal, 2 were weak, and 2 were dead. The 2 weak pigs were destroyed but no leptospiras were demonstrated either by dark-field examination or by hamster inoculation. Blood cultures in Schüffner's medium made from each of the 6 healthy pigs at birth were also negative.

Gilt 141 showed estrus 8 days after infection with *L. pomona* and 79 days after breeding. She was bred on this date and her pregnancy progressed normally to farrowing at 116 days. The litter of 7 normal pigs was nursing when found and no samples were collected.

Intravaginal Exposure to Leptospira Pomona.—Gilts 363, 394, 84, 188, 114, and 173 were exposed to *L. pomona* by depositing 1.5 ml. of culture in the vagina following breeding. Only 2 developed a febrile response, gilt 84 on the third day after exposure and gilt 188 on the sixth and seventh days. The latter also was off feed and constipated on the seventh and eighth days.

The same trend in the leukocyte counts was observed in this group of animals as the gilts exposed by the conjunctival and nasal routes. In 4 of the 6 gilts, there was an apparent leukocytosis with a corresponding increase in neutrophils.

Blood samples were collected daily during the acute phase of the disease and leptospiras were isolated from 5 of the 6 gilts. Gilt 114 was negative on all cultural attempts while the organism was recovered from the other 5 animals on one or more days (table 2).

Antibodies for *L. pomona*, demonstrated by means of the agglutination-lysis test, first appeared in all of the 6 gilts on the seventh, eighth, or ninth days with titers of 1:40 to 1:640. The peak titers, reached

TABLE 2—Observations on 6 Gilts Exposed to Leptospira Pomona by Intravaginal Injection Following Breeding

Animal (No.)	Days* of febrile response	Positive blood culture (days)	Antibody response: Day of first appearance	Antibody response: Day and level of highest titer**		Leptospiruria on day	Results of pregnancy
363	None	7	9	15	5,120	....	Farrowed normally
394	None	7	9	15	5,120	33	Dystocia, died from secondary infection
84	3	4, 6	8	18	10,240	24	Rebred twice, farrowed normally
188	6, 7	6, 7, 8	8	18	10,240	25	Farrowed normally
114	None	....	7	8	5,120	....	Rebred at 91 days, farrowed normally
173	None	5	8	15	2,560	22	Farrowed normally

* "Day" refers to the number of days following exposure to *L. pomona*.

** The titers are expressed as the reciprocal of the highest dilution of serum in which 50 per cent of the leptospiras were agglutinated or lysed.

eight to 18 days after exposure, varied from 1:2560 to 1:10,240 (table 2).

Urine samples were collected at intervals. While gilts 363 and 114 were found negative on all examinations, leptospiras were demonstrated in the urine of the other 4 between 22 and 33 days following exposure (table 2).

Gilts 363 and 394 were bred on the same day and within four hours were infected intravaginally with *L. pomona* to simulate transmission by an infected boar. The pregnancy of gilt 363 progressed normally and at 115 days she farrowed 6 normal pigs. All were nursing when found and, therefore, no samples were collected. Gilt 394 farrowed 7 living pigs at 114 days. Blood cultures from all 7 were negative and 2 were destroyed but no evidence of Leptospira was found either by dark-field examination or by hamster inoculation. During the night and the following morning, the gilt delivered 5 additional pigs, all dead. Necropsy of these pigs revealed no abnormalities and death was presumed to be related to delayed birth. The sow was weak, appeared toxic, and produced little or no milk by the second day. No treatment was given because of the probability of interference with the experiment. However, her temperature was 105 F. on the third day and 3 of the pigs were dead, so therapy with penicillin was initiated. On the fourth day the sow died, and necropsy revealed a diffuse, necrotic metritis. The cause of death was established as complications in farrowing, followed by an infection with a Clostridium sp.

Gilts 84 and 188 were exposed to *L. pomona* on the same day by the vaginal route. Gilt 84 was infected within a few minutes after breeding, was observed in estrus, and was rebred on the eighteenth day and again on the thirty-sixth day to the same boar. She farrowed, 114 days after the last breeding, 10 healthy pigs and 1 weak one which was destroyed. No leptospiras were demonstrated by direct examination or by hamster inoculation.

Gilt 188, infected 24 hours following breeding, farrowed 11 normal pigs and 1 dead pig 115 days after breeding. One of the living pigs, possibly the smallest and weakest of the litter, was destroyed but no leptospiras were demonstrated.

Gilts 114 and 173 were bred the same day and about 24 hours later each was exposed to *L. pomona* by the vaginal route. Gilt 114, 91 days after breeding, was in estrus and was rebred to the same boar. She farrowed 10 normal pigs 114 days after this breeding. All the pigs were nursing when found and, therefore, no samples were collected.

Gilt 173 farrowed 11 healthy pigs and 1 dead one 115 days after breeding. Blood cultures made from each living pig, prior to nursing, were all negative. Two of the weaker pigs were destroyed but no leptospiras were found.

Gilt 218, maintained in a separate pen adjoining those of the infected gilts, served as an unbred, uninoculated control. Intermittent serological tests were negative and at slaughter, over four months after the first animals in the building were exposed to *L. pomona*, there was no evidence of leptospirosis.

Boar 199 also served as an uninoculated control. He was kept in a separate pen in the same building with the infected gilts. He was used in natural breeding of gilts 18, 36, 79, and 91 days after they had been artificially infected. Blood cultures and tests for leptospiral antibodies were made at intervals, but six months following his first possible contact with Leptospira there was no evidence of infection.

DISCUSSION

The strain of *L. pomona* used to establish the infection in this experiment caused a mild clinical response in most of the gilts. This response was transient, usually one to four days, and probably would have been overlooked in an average farm herd. However, 4 gilts were obviously "sick and off feed."

The records of the body temperature, taken twice daily, and the blood counts, made either every day or on alternate days, supported the clinical observations. In the visibly ill animals, the febrile response continued for two or three days while in others fever was detected only at one of the two observations on one day. The blood counts revealed a leukocytosis presumably as a result of an absolute increase in the neutrophils, many of which were immature forms. Because of the amount of variation from day to day in both the total leukocyte and the differential counts for a particular animal, the use of the hemogram as a diagnostic aid does not appear to be justified.

That infection with *L. pomona* can be readily established artificially was amply demonstrated in this experiment. All of the 15 gilts which were exposed became infected. Of the 6 animals exposed by the vaginal route, 5 developed a leptospiremia between the fourth and eighth days after exposure.

The procedure used was considered to be as comparable to service by an infected boar as could be achieved artificially. Since all 6 of the gilts became infected, presumably through the unbroken mucosa of the vagina or possibly of the uterus, it seems likely that breeding by a boar that is shedding Leptospira in the urine would serve as a source of infection. It would be of value to repeat this type of experiment with infected boars in order to establish this point.

Limited observations were made on the possibility of spreading infection from the sow to the boar at breeding. Boar 199 served 3 infected sows but there was no evidence that he became infected. Of particular interest is gilt 84, infected intravaginally immediately following breeding by boar 199, who was in estrus 18 and 36 days later and was rebred by boar 199 on both occasions. On the twenty-fourth day following infection, *L. pomona* was isolated from her urine. Boar 199 also served gilts 114 and 141 at 90 days and eight days postinfection, respectively. Leptospiras were never demonstrated in the urine of gilt 114. It is unlikely that localization of the organism in the kidney and the shedding in the urine could have occurred by the eighth day in gilt 141. However, the contact with gilt 84 would seem to have been an ample opportunity for infection of the boar.

Intermittent shedding of Leptospira in the urine has been observed, and it is possible that there were no leptospiras being shed by gilt 84 on the days of breeding. Thus, even though boar 199 was in contact with infected sows on 4 occasions it is possible that he was not actually exposed to viable Leptospira. This boar was not challenged prior to slaughter.

The necessity of direct contact with infected urine of the carrier is further emphasized by the fact that a control gilt (218) was maintained in an individual pen adjoining those containing infected swine throughout the experiment without becoming infected. No special precautions were taken by the persons during feeding and watering, and cleaning of the pens. Nevertheless, when sent to slaughter, this gilt showed no signs of leptospirosis and no antibodies were demonstrated.

The fact that 4 of the 6 gilts exposed by the vaginal route shed Leptospira in their urine, whereas only 2 of the 9 gilts exposed conjunctivally and nasally were urinary shedders, may be of significance. However, since a leptospiremia followed in most of the animals, regardless of the route of infection, it may be that the portal of entry is of little importance. Certainly, the essentially normal pregnancy in each of the 6 gilts exposed intravaginally would indicate that the proximity of the portal of entry to the uterus had no special influence on causing abortion.

Leptospirosis was produced readily in 9 gilts by the conjunctival and nasal routes, although either route alone would probably have been sufficient.[4] The leptospiras are obviously capable of penetrating the conjunctiva or the mucous membrane of the respiratory tract. Since infected urine is the most important source of the infection, the probable portal of entry in a natural spread of leptospirosis in a herd would be by the splashing of urine in the eyes or by the susceptible pig rooting or smelling recently voided urine.

Even though 100 per cent of the animals exposed in this experiment became infected and most of the gilts were at least mildly ill clinically, there was relatively little effect on the progress of pregnancy. Of the 6 gilts exposed intravaginally, 3 completed normal pregnancies. Of the other 3, gilt 394 died after farrowing from parturient complications, and gilt 84 required two rebreedings before conception. In neither of these cases could the abnormality be associated with leptospirosis. The third gilt (114) was presumed to be pregnant but was in estrus 91 days after breeding. No evidence of abortion had been observed and she delivered a normal litter from a breeding on this day, suggesting that there was no abnormality in the uterus. Gilt 114 was never observed shedding Leptospira in the urine but the possibility still exists that there was an early abortion or resorption due to infection from *L. pomona*.

All 3 gilts exposed to *L. pomona* during the first month of pregnancy farrowed

normally, as did 2 of the 3 exposed in the third month of pregnancy. None of the 3 gilts infected 48 to 53 days after breeding farrowed. Gilt 233 aborted at 86 days and the other 2, although presumably pregnant, were not seen in estrus and did not farrow. In neither case was there any evidence of abortion but, during a part of this period, each of the gilts was penned with 1 other sow so it is possible that small immature fetuses may have been eaten. The other gilt (141) was observed in estrus 79 days after breeding and eight days after infection. She was rebred and farrowed a normal litter. There was no evidence of abortion in this gilt and, in fact, with estrus appearing so early after exposure to *L. pomona*, the role of the infection in causing this reproductive failure is questionable.

In reviewing the effect of the leptospiral infection on these pregnancies, one can only conclude that there was relatively little damage. Only 1 sow aborted; in 3 others the infection may have caused a reproductive failure. That *L. pomona* can cause extensive losses from abortion in field outbreaks has been reported by several workers.[1-3]

Ryley and Simmons [5] reported results of an experiment in Australia which are in contrast with our observations. Four sows infected intramuscularly with *L. pomona* in the second half of pregnancy produced 37 pigs of which 34 were dead or died within a few minutes of birth. The average gestation was 95 days. Four control sows maintained under similar conditions farrowed, at 115 days, 35 pigs of which 29 lived. Another difference is that little clinical evidence of acute leptospirosis was reported by Ryley and Simmons. Three of their sows remained essentially normal while the fourth did respond with a fever of over 105 F. and showed some dullness and anorexia.

These observations raise the question of whether there is any correlation between the virulence of *L. pomona* for swine as indicated by clinical signs and the effect on the pregnant sow. This difference in the results produced by the infection might be related to the strain of *L. pomona* used in the exposure. In our experiment, the infecting organism was isolated originally from a field outbreak in which only 79 pigs survived of 257 farrowed by 25 sows. The culture was maintained for less than one year in guinea pigs, hamsters, and, intermittently, on an artificial medium. It was passed serially through 3 pigs just prior to use in this experiment. The strain used by Ryley and Simmons [5] was originally isolated from swine and the inoculum for the experimental sows consisted of culture from the twenty-fifth guinea pig passage. The history of the organism used in experimental leptospirosis may prove to be of considerable importance. Work now in progress in our laboratory indicates that virulence of a particular strain of *L. pomona*, as measured by the severity of the acute phase of the disease, may differ considerably in the hamster and in cattle.[6]

The status of pregnancy at the time of exposure to *L. pomona* may be of considerable importance in determining the effect of the infection on the reproductive organs. The results reported in this paper indicate that sows near mid-term at the time of exposure may be more likely to abort than those in early or in late pregnancy.

SUMMARY AND CONCLUSIONS

Fifteen gilts were artificially infected with *Leptospira pomona*. The 9 exposed conjunctivally and nasally at different stages of pregnancy developed various degrees of clinical signs of acute leptospirosis. Three gilts exposed during the second month of pregnancy appeared to be at the most susceptible stage since none of these farrowed a normal litter, while infection in the first and third months of pregnancy seemed to have little effect on reproduction.

No reproductive failure definitely attributed to leptospirosis was observed in the 6 gilts infected intravaginally immediately after breeding.

In view of the results of this experiment, of other controlled experiments cited, and of naturally occurring leptospirosis observed in swine, it is concluded that the strain of *L. pomona* producing the infection may be important in determining the effect on the reproductive organs. The virulence, as measured by the severity of the acute phase of the disease, may not be closely associated with the abortifacient activity. The stage of pregnancy at the time of the first exposure to *L. pomona* is undoubtedly an important factor in deter-

mining whether abortion or reproductive failure occurs.

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Studies on Infectious Atrophic Rhinitis. V. Concept that Several Agents May Cause Turbinate Atrophy

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SINCE turbinate atrophy in swine was first reported in 1830, numerous investigators have concluded that it resembled an infectious process but were unable to transmit it. Jones [1] showed that the critical factor in transmission was the age of the inoculated pig. Since this finding, many investigators have succeeded in transmitting turbinate atrophy. A tendency has developed to think of this disease, which is called infectious atrophic rhinitis, as being a specific infectious disease. It is the purpose of this paper to present evidence indicating that different agents may cause turbinate atrophy.

MATERIALS AND METHODS

The pigs used in these experiments were from an isolated herd maintained at the Veterinary Medical Research Institute as a source of experimental pigs. In the initial stages of this work, this herd was found to produce occasional pigs that, when reared by the sows, developed pneumonia and turbinate atrophy. Therefore, a new herd was developed from stock taken by hysterectomy by Dr. G. A. Young at the Hormel Institute and by cesarean section by the author at this Institute and reared in isolation. The new herd remained separate from the old herd which was sold as quickly as replacement could be accomplished. Necropsies were done on most of the pigs reared in the new herd. No turbinate atrophy or pneumonia has been observed in pigs reared by the dams in this herd. No *Pasteurella multocida*, *Mycoplasma hyorhinis* (the swine pleuropneumonia-like organism), or *Hemophilus suis* have been recovered to date from this new herd. Approximately half of the pigs used in this experiment were from this new herd.

When the sows were moved into the farrowing house, they were scrubbed with warm water and detergent then housed in individual pens that had been scrubbed with 2 per cent lye solution. The pigs were removed from the sow when a few hours old and taken to disinfected isolation units.

The isolation rooms opened to the outside and each had four pens separated by wire partitions. The pigs were fed fresh concentrated cow's milk (12 % butterfat) diluted with an equal amount of water. Vitamins A and D and 5 ml. of the mineral mixture described by Young [2] were added to each quart of the diluted milk. The pigs were fed three times daily for ten days and two times daily thereafter. Their caretaker had no other contact with swine.

When possible, a portion of each litter was maintained as uninoculated controls. Initially, the control pigs were inoculated intranasally with sterile tryptose broth but since this was found to produce no lesions it was not done in later experiments.

Approximately 30 litters were obtained from the old herd and reared by this method. No evidence of turbinate atrophy or pneumonia was observed in the control pigs except in one case. In this instance, the bacterial culture given the inoculated group was apparently transferred to the control group and both groups developed turbinate atrophy from which the test organism was isolated.

Inoculations, on each of four to six days, were usually started when the pigs were 1 or 2 days of age and in all cases before the fourth day. Approximately 1 ml. of inoculum was given intranasally with the pig held on its back with its head extended. The base of the syringe sealed the external nares while the inoculum was administered.

The number of pigs used in each trial was determined by the number in the litter and the isolation units available. Whenever possible 3 or 4 pigs were given each inoculum. Several trials were not reported because either the control or the inoculated pig died of enteritis during the first several days of life. Some pigs, which died of enteritis during the course of other trials, were not included in the report of these trials.

Most of the trials were terminated when the pigs were destroyed for necropsy, when 4 to 6 weeks old. Turbinate atrophy was evaluated by gross examination of a cross section of the ventral turbinate in the region of maximum development of the dorsal and ventral scroll. This is usually near the level of the first premolar tooth. To accomplish this, the head was split longitudinally along the nasal septum, the septum removed, and the ventral turbinate detached. Sharp scissors were used for this and for removing a cross section of the turbinate in the area of maximum scroll development. This procedure produced less distortion of the turbinate than when a cross section of the nasal cavity was made with a saw.

Bacterial cultures, prepared from the nasal tur-

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Portions of this work have been reported at the 1953, 1954, and 1955 meetings of the NC-13 committee (death losses of baby pigs).

binates, were secured from the surface of the posterior half of the turbinate, since it was repeatedly observed that in normal nasal turbinates bacteria were usually present on the surface of the anterior third, seldom on the middle third, and rarely on the posterior third. The inoculated blood agar plates were incubated aerobically. A culture of Micrococcus sp. was streaked on the surface of the cultures made to determine the presence of *H. suis*.

Isolation of *Mycoplasma hyorhinis* from the nasal cavities of experimental pigs was accomplished either by embryo inoculation or by inoculation of ox heart infusion-chicken serum medium as described previously.[4]

A second small herd of swine affected with turbinate atrophy was maintained in isolation. The pigs were infected, during the first few days of life, by repeated intranasal inoculation with crude atrophic turbinate suspension obtained from older pigs in the herd. In addition, some inoculum was obtained from field herds affected with turbinate atrophy and pneumonia.

When necessary to store material for more than a few hours, it was placed in screw-top glass containers and frozen at approximately —40 C.

Cellulose membrane filtrates were prepared using cellulose ultrafilters.* Approximately 5 per cent lung or 2 per cent turbinate suspensions in tryptose broth were frozen at —40 C., thawed, centrifuged at 2,000 r.p.m. for 15 minutes, filtered through either a Selas filter candle or cellulose membrane at pressures of 8 to 20 inches of mercury. The use of the Selas No. 015 filter to clarify the suspension prior to filtration through the 0.5 to 0.75 μ cellulose membrane simplified filtration, but titration trials to determine that the Selas filters did not reduce the titer of the inoculum, were not conducted. Unless specified to the contrary, all filtrates used were bacteriologically negative when cultured on blood agar plates and incubated aerobically for 48 hours.

It was found that Selas filters No. 01 and No. 015 would frequently produce filtrates free of bacteria if the original material was not heavily contaminated. The same was true of the 0.75 to 1.0 μ cellulose membrane. It was found that the 0.5 to 0.75 μ cellulose membranes would usually produce bacteria-free filtrates even from heavily contaminated material. This membrane was observed to produce sterile filtrates when tryptose broth cultures of *P. multocida* and Alcaligenes sp. were filtered through it. However ox heart infusion-chicken serum cultures of *Mycoplasma hyorhinis* were not sterile after filtration through 0.5 to 0.75 μ cellulose membrane.

Results

The results obtained in this series of inoculation trials are summarized in table 1.

A Selas No. 01 filtrate and a Selas No. 015 filtrate were prepared from the atrophic turbinate suspension obtained from a pig with grossly normal lungs from the infected herd. At necropsy six weeks after the first inoculation, turbinate atrophy was present in 2 of 3 pigs (trial 1) receiving the Selas No. 01 filtrate which contained bacteria, while all 3 pigs receiving the Selas No. 015 filtrate (trial 2) had turbinate atrophy. All 6 pigs had normal appearing lungs. The 3 control pigs for these two trials had normal appearing lungs and turbinates.

The trial with the Selas No. 015 filtrate was repeated on 7 pigs (trial 3) using atrophic turbinates from 2 pigs from a field case of turbinate atrophy and pneumonia. All 7 had turbinate atrophy and 3 of 7 had small areas of pneumonia at necropsy five weeks after the first inoculation; all 8 control pigs had normal appearing lungs and turbinates.

In an attempt to consistently produce filtrates containing the atrophy-producing agent(s) free of bacteria, a 0.75 to 1.0 μ porosity cellulose membrane filtrate was prepared from a pool of atrophic turbinates collected from pigs from three different herds affected with pneumonia and turbinate atrophy. Three of 4 pigs inoculated with this filtrate (trial 4) had turbinate atrophy four weeks after the initial inoculation and 3 of the 4 had pneumonia. The 4 control pigs had normal lungs and turbinates.

Cellulose membranes of 0.75 to 1.0 μ and 0.5 to 0.75 μ porosity were used to prepare filtrates of a suspension of atrophic turbinates from a pig with turbinate atrophy and pneumonia. Both of 2 pigs inoculated with the 0.75 to 1.0 μ membrane filtrate (trial 5) had turbinate atrophy and normal appearing lungs while 2 of 3 pigs inoculated with the 0.5 to 0.75 μ membrane filtrate (trial 6) had turbinate atrophy with no pneumonia five weeks after the first inoculation. The 3 control pigs had normal lungs and turbinates.

Bacteria-free filtrates could be prepared from atrophic turbinate specimens from the isolated infected herd and from two other herds that produced turbinate atrophy but not from all infected herds. One such specimen was obtained from a pig with severe turbinate atrophy associated with distortion of the nose. The turbinate of this pig yielded a pure culture of *P. multocida*. This crude turbinate suspen-

* Carl Schleicher and Schuell Co., Keene, N. H.

TABLE 1—Summary of Inoculation Results in the Study of Infectious Atrophic Rhinitis

Trial No.	Source of infected pigs	Lesions in infected pig		Tissue used for inoculum	Preparation of inoculum	Lesions in experimental pigs			
						Inoculated		Control	
		Turbinate atrophy	Pneumonia			Turbinate atrophy	Pneumonia	Turbinate atrophy	Pneumonia
1	VMRI* infected herd	+	—	Turbinate	Selas No. 01 filter	2/3	0/3	0/3	0/3
2	Same pig as trial 1	+	—	Same tissue as trial 1	Selas No. 015 filter	3/3	0/3		
3	Field herd A	+	+	Turbinate	Selas No. 015 filter	7/7	3/7	0/8	0/8
4	VMRI infected herd, field herds B and C	+	+	Turbinate	0.75–1.0 μ cellulose membrane	3/4	3/4	0/4	0/4
5	VMRI infected herd	+	+	Turbinate	0.75–1.0 μ cellulose membrane	2/2	0/2	0/3	0/3
6	Same pig as trial 5	+	+	Same tissue as trial 5	0.5–0.75 μ cellulose membrane	2/3	0/3		
7	Field herd D	+	¶	Turbinate	Crude turbinate	2/3	2/3	No controls	
8	Same pig as trial 7	+	¶	Same tissue as trial 7	0.5–0.75 μ cellulose membrane	0/3	0/3	0/2	0/2
9	Same pig as trial 7	+	¶	.....	*Pasteurella multocida* from atrophic turbinate	2/4	2/4	No controls	
10	Inoculated pig from trial 9	+	+	.....	*P. multocida* from atrophic turbinate	0/3	0/3	No controls	
11	VMRI infected herd	+	+	.....	Guinea pig lesions produced by Alcaligenes sp.	2/2	1/2	2/2†	0/2
12	Same pig as trial 11	+	+	.....	Alcaligenes sp. cultured from atrophic turbinate	3/3	1/3	0/2	0/2
13	Inoculated pig from trial 12	+	+	.....	Alcaligenes sp. cultured from atrophic turbinate	2/2	0/2	0/2	0/2
14	Field herd E	+	¶	.....	*Hemophilus suis* cultured from atrophic turbinate	0/3	0/3	No controls	
15	Field herd F	+	¶	.....	*Pseudomonas aeruginosa* from atrophic turbinate	0/3	0/3	No controls	
16	.....	....	....	.....	1½ % acetic acid	3/4	0/4	0/3	0/3
17	.....	....	....	.....	2 % + 4 % acetic acid	2/4	0/4	0/2	0/2
18	Field herd G	+	+	Pneumonic lung	Crude lung	1/1	1/1	0/1	0/1
19	Same pig as trial 18	+	+	Same tissue as trial 18	Crude lung	4/4	0/4	0/4	0/4
20	VMRI infected herd	+	+	Turbinate	Crude turbinate	5/5	3/5	No controls	
21	Same pig as trial 20	+	+	Same tissue as trial 20	Crude turbinate + 20,000 units penicillin + 1:4,000 thallous acetate/ml.	3/4	1/4	No controls	
22	Field herd H	+	+	Pneumonic lung	Crude lung + 8,000 units penicillin + 1:4,000 thallous acetate/ml.	4/4	3/4	0/3	0/3
23	Inoculated pig from trial 22	+	+	Pneumonic lung	Crude lung + 5,000 units penicillin + 1:4,000 thallous acetate + 5 mg. terramycin® ml.	0/4	0/4	No controls	
24	Inoculated pig from trial 22	+	+	Same tissue as trial 23	Crude lung + 5,000 units penicillin + 1:4,000 thallous acetate/ml.	3/4	0/4	No controls	

* Veterinary Medical Research Institute; ¶ not available; † Alcaligenes sp. isolated from these atrophic turbinates; denominator—No. inoculated; numerator—lesions present.

sion produced turbinate atrophy and pneumonia in 2 of 3 inoculated pigs (trial 7) five weeks after the first inoculation. A 0.5 to 0.75 μ cellulose membrane filtrate of this turbinate suspension failed to produce turbinate atrophy (trial 8) in any of 3 inoculated pigs. The lungs of these pigs appeared normal. The 2 control pigs appeared normal.

The *P. multocida* culture isolated from the atrophic turbinate was transferred from the original blood agar plate into tryptose broth. A 0.2-ml. portion of the 24-hour tryptose broth culture was inoculated into chicken embryos. Amnio-allantoic fluid from the chicken embryos dead 24 to 48 hours postinoculation was used to inoculate 4 baby pigs (trial 9). Two of the 4 pigs had turbinate atrophy five weeks after the first inoculation. *Pasteurella multocida* was recovered from the nasal cavities of all 4, and 3 of the 4 pigs had small areas of pneumonia.

The *P. multocida* isolated from one of the atrophic turbinates in the previous trial was grown in tryptose broth. Twenty-four to 72-hour cultures of the fourth to seventh transfers were inoculated intranasally into 3 pigs (trial 10). Six weeks after their first inoculation, their turbinates and lungs appeared normal. *Pasteurella multocida* was not recovered from their turbinates.

An Alcaligenes sp. resembling *Alcaligenes faecalis* was recovered from an atrophic turbinate. This organism produced a severe peritonitis and scrotal inflammation in a male guinea pig. This guinea pig lesion material produced turbinate atrophy (trial 11) in 2 of 2 inoculated pigs and a small area of pneumonia in 1. The 2 control pigs housed in the isolation unit next to the inoculated pigs also had turbinate atrophy. Alcaligenes sp. was isolated from their turbinates as well as from those of the inoculated pigs. A 24-hour tryptose broth culture of the sixth to tenth transfer of this organism, when inoculated into 3 pigs (trial 12), produced severe turbinate atrophy in 3 and a small area of pneumonia in 1 of these pigs. The organism was recovered from the turbinates of all 3 pigs. The 2 control pigs had normal appearing turbinates and lungs and bacterial cultures from their turbinates were negative.

Two pigs (trial 13) were inoculated with a 24-hour tryptose broth culture of the fourth to eighth transfers of the Alcaligenes sp. isolated from the atrophic turbinates of 1 of the inoculated pigs in trial 12. Both pigs had atrophic turbinates five weeks after the first inoculation and Alcaligenes sp. was recovered from each. The lungs of these animals appeared normal. The 2 control pigs had normal turbinates and lungs. No bacteria were isolated from their turbinates.

Three pigs (trial 14) were inoculated intranasally with 24- to 48-hour cultures of the sixth to tenth transfer in ox heart-chicken serum medium of a *H. suis* isolated in essentially a pure culture from the atrophic turbinate of a pig from a field case. Five weeks after the first inoculation their turbinates and lungs appeared normal, but *H. suis* was isolated from the turbinates of all 3 pigs.

An abundant growth of *Pseudomonas aeruginosa* was isolated from the atrophic turbinates of a pig from a field case. Twenty-four to 72-hour cultures of the third to sixth passage of this organism in tryptose broth were inoculated intranasally into 3 pigs (trial 15). These pigs had normal appearing turbinates and lungs six weeks after the first inoculation and *Ps. aeruginosa* was not isolated from their nasal turbinates.

Since a filter-passing agent(s) and two unrelated bacteria were found capable of producing turbinate atrophy, it was decided to investigate the possibility that prolonged irritation of the nasal cavity by noninfectious material would also produce turbinate atrophy. Acetic acid was used as the irritant (trial 16). Approximately 1 ml. of 1.5 per cent acetic acid was introduced into the nasal cavities on 30 of the 35 days this trial was conducted. Severe sneezing was observed. Three of 4 treated pigs developed definite turbinate atrophy and the fourth pig had considerable nasal exudate. The lungs of these pigs were normal. The 3 control pigs had normal lungs and turbinates.

Since the irritating effect of the acetic acid appeared to decrease after the pigs were 2 weeks of age, the trial was repeated (trial 17) introducing 2 per cent acetic acid for the first ten days and 4 per cent acetic acid thereafter. The 4 pigs in this trial sneezed severely after the third treatment. They were treated on 30 of the 38 days this trial was conducted. Two of the 4 had turbinate atrophy at necropsy but the lungs of all 4 appeared normal. The 2

control pigs had normal turbinates and lungs.

Pneumonia is frequently present in field cases of turbinate atrophy. Some of these pneumonic lesions failed to yield bacterial growth when cultured aerobically on blood agar. One such specimen was collected from a pig with turbinate atrophy, stored at —40 C. for six weeks, and inoculated into 1 normal pig (trial 18). This pig had turbinate atrophy and pneumonia five weeks after the first inoculation. No *P. multocida* were recovered from the atrophic turbinate or the pneumonic lung. The control pig had normal lungs and turbinates.

This trial was repeated (trial 19) using another portion of the same inoculum after seven weeks' storage at —40 C. All of 4 inoculated pigs had turbinate atrophy but no pneumonia five weeks after the first inoculation. No *P. multocida* were recovered from the atrophic turbinates. The 4 control pigs had normal lungs and turbinates.

Since penicillin and thallous acetate can be used to inhibit many bacteria, it was decided to determine what effect these drugs had on the production of turbinate atrophy by a crude atrophic turbinate suspension obtained from the isolated infected herd. Atrophic turbinates from this herd had failed to yield *P. multocida* but turbinate suspensions had yielded atrophy-producing filtrates after filtration through Selas No. 015 filters or 0.5 to 0.75 μ cellulose membranes. An untreated crude atrophic turbinate suspension was prepared from a pig of this herd and was found to produce turbinate atrophy (trial 20) in 5 of 5 inoculated pigs four weeks after the first inoculation. Three of these 5 pigs had pneumonia. Material from the same crude turbinate suspension was treated with 20,000 units of crystallin penicillin per milliliter and enough thallous acetate to make a 1:4,000 concentration. The treated material remained at room temperature for one hour prior to each inoculation into 4 pigs (trial 21). Three of the 4 pigs showed turbinate atrophy four weeks postinoculation, and 1 pig also had a small area of pneumonia. It was not possible to arrange for control pigs for this trial.

A suspension of pneumonic lung was secured from a pig with a field case of turbinate atrophy and pneumonia. This suspension was stored at —40 C. for ten days, then treated with 8,000 units of crystallin penicillin and sufficient thallous acetate to make a 1:4,000 concentration. When 4 pigs (trial 22) were inoculated with this material, four to six weeks postinoculation all 4 showed turbinate atrophy. In addition, 3 of the 4 had pneumonia. The 3 control pigs had normal turbinates and lungs.

Part of this same pneumonic lung suspension was stored at —40 C. for 15 days, treated with 5,000 units of penicillin, thallous acetate sufficient to make a 1:4,000 concentration and 5 mg. of terramycin® per milliliter, allowed to stand at room temperature 30 minutes, and then was stored at —40 C. Six weeks after 4 pigs were inoculated with this material (trial 23), the turbinates and lungs of all 4 appeared normal. A group of 4 pigs (trial 24) was inoculated with similar material except that it did not contain terramycin. Three of the 4 had turbinate atrophy five weeks after the inoculation. The lungs of these pigs appeared normal.

DISCUSSION

In a previous article [2] it was reported that a Selas No. 02 filtrate of atrophic turbinates produced turbinate atrophy in 1 of 3 inoculated pigs; that all 4 control pigs had normal turbinates and lungs; and that an inoculation of Selas No. 10 filtrate from the same crude atrophic turbinate suspension resulted in turbinate atrophy in all of 3 pigs six weeks postinoculation. In addition 1 of the 3 pigs had pneumonia. Two other inoculation trials, conducted with Selas No. 02 filtrates of atrophic turbinate material from this same isolated, infected herd failed to produce turbinate atrophy or pneumonia in six weeks in 3 of 3 inoculated pigs in the second trial or in 2 of 2 inoculated pigs in the third trial. Since the Selas No. 02 filter used to prepare the filtrate was found to have a bubbling pressure less than specified for a No. 02 filter, larger pore size filtrates of atrophic turbinates of pigs were prepared and tested. The results obtained in these trials indicate that there is present in certain atrophic swine turbinates an agent(s) that will pass a Selas No. 015 filter of a cellulose membrane of 0.5 to 0.75 μ pore size.

It appears that the filter-passing, atrophy-producing agent(s) is distinct from *Mycoplasma hyorhinis* although the latter will pass through smaller pore size filters than the atrophy-producing agent(s). It

has been found [2] that *Mycoplasma hyorhinis* does not produce turbinate atrophy even though it becomes established in the nasal cavity. Results of attempts to isolate this organism from the nasal cavities of the pigs used in the present work are summarized (table 2). It apparently occurred in the nasal cavities of swine with either atrophic or normal turbinates. In addition, it appears that in some trials the filter-passing agent(s) produced turbinate atrophy in the absence of *Mycoplasma hyorhinis*.

In several of the trials, the pigs became infected with *Mycoplasma hyorhinis* during the few hours they remained with the dam.

The filter-passing turbinate atrophy agent(s) occurs in some pneumonic lung specimens from swine with turbinate atrophy. It appears that this agent(s) can be stored at —40 C. up to seven weeks and that crystallin penicillin and thallous acetate do not inhibit it at the usual levels employed for bacterial inhibition. However terramycin appeared to inhibit the agent(s) (trial 23). The agent(s) present in one atrophic turbinate (trial 21) and one pneumonic lung specimen (trial 22) that produced pneumonia in the inoculated pigs also appeared to withstand penicillin and thallous acetate at the levels usually employed to inhibit bacteria.

It is of interest that both turbinate atrophy and pneumonia were produced (trials 3 and 4) when the two atrophic turbinate filtrates from field herds were used, but turbinate atrophy alone was produced in four filtrate trials (1, 2, 5, and 6) which utilized atrophic turbinate material from the closed infected herd maintained for this work. The results indicate the possibility that some filtrates of atrophic turbinates contain agent(s) capable of producing only turbinate atrophy while other specimens contain agents capable of producing both turbinate atrophy and pneumonia.

In addition, in two trials (16 and 17) acetic acid produced turbinate atrophy but no pneumonia. This suggests that in the two filtrate inoculation trials, in the two treated crude inoculum trials, and in some of the Alcaligenes sp. and *P. multocida* trials the pneumonia was not due to inhalation of foreign material. It seems more likely that the pneumonia was produced by some agent(s) present in the inoculum.

Failure of *P. multocida* (trial 10) to produce turbinate atrophy upon reisolation from atrophic turbinates points out the problem of maintaining the virulence of organisms under investigation. For this reason, failure of *H. suis* and *Ps. aeruginosa* cultures to produce turbinate atrophy can not be interpreted as indicating that adequately virulent cultures of these organisms would not produce turbinate atrophy.

TABLE 2—Summary of Mycoplasma Hyorhinis Isolation from Nasal Turbinates

Trial No.	Inoculated animals		Control animals	
	Turbinates atrophied	*M. hyorhinis* recovered	Turbinates atrophied	*M. hyorhinis* recovered
1	2/3	3/3	0/3	¶
2	3/3	3/3		
3	7/7	7/7	0/8	Pool of 8
4	3/4	2/4	0/4	0/4
5	2/2	2/2	0/3	3/3
6	2/3	3/3		
7	2/3	3/3	No controls	
8	0/3	0/3	0/2	0/2
9	2/4	4/4	No controls	
10	0/3	0/3	No controls	
11	2/2	0/2	2/2*	0/2
12	3/3	0/3	0/2	0/2
13	2/2	0/2	0/2	0/2
14	0/3	0/3	No controls	
15	0/3	0/3	No controls	
16	3/4	0/4	0/3	0/3
17	2/4	0/4	0/2	0/2
18	1/1	0/1	0/1	0/1
19	4/4	2/4	0/4	2/4
20	5/5	3/5	No controls	
21	3/4	4/4	No controls	
22	4/4	2/3†	0/3	0/3
23	0/4	0/4	No controls	
24	3/4	0/4	No controls	

¶ Turbinates not cultured; † 1 not cultured; * Alcaligenes sp. isolated from these atrophic turbinates.

There was a difference in time interval before signs of disease between pigs inoculated with filtrates free of bacteria and pigs inoculated with bacterial cultures which produced turbinate atrophy. The pigs receiving the bacteria-free filtrates usually did not sneeze or sniffle until about the third week after the first inoculation. In one positive trial, no unusual sniffling or sneezing was noticed during the entire observation period. However, the pigs receiving bacterial cultures (and those treated with acetic acid) usually began to sniffle before the course of treatment was completed, with sneezing and sniffling at a maximum in about two to four weeks after which they sometimes began to subside.

The lesions observed in the atrophic turbinates produced by the various measures reported above can be grouped into two classes and briefly summarized as follows: (1) The bacteria-free filtrates and treated crude inoculum usually produced a minimum of nasal exudate. In some cases, there appeared to be no grossly visible increase in nasal exudate; (2) the bacterial inoculums and acetic acid usually produced a turbinate atrophy with considerable mucous and mucopurulent exudate on the surface of the turbinates. This exudate frequently contained flecks of desquamated epithelium. The ventral scroll of the ventral turbinate was usually the site of the most severe atrophy in both the bacteria-free filtrate-produced and bacteria or acetic acid-produced atrophy.

There appeared to be a difference in the microscopic changes observed in sections of atrophic turbinates resulting from instillation of the filter-passing atrophy-producing agent(s) and of bacterial cultures or acetic acid. The chemical and bacterial materials appeared to produce considerable irritation of the surface of the turbinate with resultant inflammatory reaction but had little visible effect on the osteoblasts. It appeared as though the reduced size of the turbinate resulted from its failure to grow at a normal rate. The filter-passing agent(s) appeared to produce little alteration of the epithelium of the nasal turbinate but resulted in considerable infiltration of the submucosa with lymphocytes and lymphoblasts. There sometimes was a marked increase in the number of osteoblasts. In some cases, the osteocytes and osteoblasts appeared to be dedifferentiating into tissue resembling fibrous connective tissue with a resultant resorption of the bone salts. In some of these cases, this band of tissue had replaced whole areas of the normal turbinate bone.

The characteristics of the filter-passing agent(s) are not yet sufficiently defined to indicate the class of microorganisms to which it belongs. It is possible that it is a very large virus, a pleuropneumonia-like organism (not *Mycoplasma hyorhinis*), or a small rickettsia. However, numerous smears from atrophic turbinates and pneumonic lungs have been stained with Giemsa's stain and examined with no conclusive evidence of the presence of rickettsia, although some preparations have been difficult to evaluate.

The production of turbinate atrophy by such widely divergent groups of agents as filter-passing agent(s), two unrelated bacteria, and acetic acid point out the nonspecificity of turbinate atrophy in swine.

SUMMARY

Selas No. 015 filter or cellulose membranes of 0.5 to 0.75 μ pore size produce filtrates of some atrophic turbinate suspensions containing an agent(s) that produces turbinate atrophy. Two of the filtrates also produced pneumonia in the experimental pigs. Some pneumonic lung and atrophic turbinate specimens contained an agent(s) capable of producing turbinate atrophy and, in two cases, pneumonia, after treatment with penicillin and thallous acetate. Not all atrophic turbinates contained this agent(s). *Pasteurella multocida* and Alcaligenes sp. were recovered from some atrophic turbinates and found to produce turbinate atrophy. Acetic acid instillations were also found to produce turbinate atrophy. These findings suggest that several unrelated agents may cause turbinate atrophy in swine.

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Effect of Repeated Freezing and Thawing on the Electrophoretic Pattern of Swine Serum

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IT HAS BEEN observed that sharp freezing and rapid thawing of mammalian serum results in the formation of a quantity of insoluble precipitate. Swine serum is particularly prone to exhibit this effect. Frequently serum samples submitted to this laboratory for electrophoretic examination are too numerous for analysis in a single day; those serums not immediately analyzed are routinely frozen and stored in an electric deep freeze at —20 C. The period of storage may vary from a few days to as long as several months. This investigation was initiated to determine the possible effect of freezing and thawing on the electrophoretic pattern of swine serum proteins. No attempt was made to characterize the precipitate.

MATERIALS AND METHODS

Serum.—Blood was obtained aseptically by venapuncture from a 6-month-old, healthy animal vaccinated against hog cholera. Serum was separated by clotting and centrifugation. No preservatives were added to the serum. After separation, a sample of serum was removed for analysis and the remainder immediately frozen in an electric deep freeze at —20 C. Subsequent electrophoretic analyses were made on the serum at prescribed intervals for 26 weeks (table 1). The container of frozen serum was immersed in water at 37 C. until completely thawed, then thoroughly mixed by gentle agitation to evenly distribute the precipitate. After each sampling, the remaining serum was refrozen. Each sampling was processed the day it was taken.

Analysis.—Each sampling of the serum was diluted with four volumes of phosphate buffer, pH 7.6, and dialyzed at 4 C. against the same buffer for sixteen hours. After dialysis the remaining precipitate was removed by centrifugation. A single analysis of each sampling was made in an Aminco portable electrophoresis apparatus using a current of 26 ma for two hours. Final patterns were recorded photographically. Ascending patterns were enlarged, minimum ordinates constructed, and areas measured planimetrically. From these areas, percentages of serum components were calculated.

RESULTS AND DISCUSSION

The results of repeated electrophoretic analyses on a serum after each of 12 freeze-thaw cycles are presented in table 1. The initial analysis showed 51.0 per cent albumin, 15.4 per cent *alpha* globulin, 15.7 per cent *beta* globulin, and 17.9 per cent *gamma* globulin. After 12 freeze-thaw cycles, the distribution of the 26-week sample was 50.5 per cent albumin, 15.4 per cent *alpha* globulin, 15.8 per cent *beta* globulin, and 18.4 per cent *gamma* globulin. The arithmetic average of the 13 analyses

TABLE 1—Analysis of a Serum After Repeated Freezing and Thawing

Time (weeks)	Albumin		*Alpha* Globulin		*Beta* Globulin		*Gamma* Globulin	
	(%)*	(D)**	(%)*	(D)**	(%)*	(D)**	(%)*	(D)**
0	51.0	0.5	15.4	0.0	15.7	0.0	17.9	0.4
1	48.5	3.0	13.8	1.6	15.9	0.2	21.8	4.3
2	52.4	0.9	15.2	0.2	15.8	0.1	16.7	0.8
3	49.7	1.8	16.5	1.1	16.2	0.5	17.6	0.1
4	53.0	1.5	15.1	0.3	15.3	0.4	16.5	1.0
5	53.5	2.0	15.6	0.2	14.5	1.2	17.5	0.0
6	53.5	2.0	15.2	0.2	15.5	0.2	15.7	1.8
8	54.7	3.2	14.1	1.3	15.9	0.2	15.3	2.2
10	52.0	0.5	13.1	2.3	17.9	2.2	16.9	0.6
12	54.2	2.7	15.8	0.4	14.5	1.2	15.6	1.9
14	46.4	5.1	19.8	4.4	16.0	0.3	17.7	0.2
20	50.5	1.0	14.8	0.6	15.0	0.7	19.8	2.3
26	50.5	1.0	15.4	0.0	15.8	0.1	18.4	0.9
Av.	51.5	1.9	15.4	1.0	15.7	0.6	17.5	1.3

* Expressed as percentage of the total area; ** deviation from the arithmetic average.

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was 51.5 per cent albumin, 15.4 per cent *alpha* globulin, 15.7 per cent *beta* globulin, and 17.5 per cent *gamma* globulin. The average values compare favorably with the percentages found for the serum originally and following the final freeze-thaw cycle. These data demonstrate that repeated freezing and thawing produces no significant alteration in the electrophoretic pattern of swine serum.

SUMMARY

A sample of swine serum was subjected to 12 freeze-thaw cycles covering a 26-week period. Data presented demonstrate that no significant alteration occurred in the electrophoretic pattern of serum proteins after repeated freezing and thawing. Variations were noted in individual samplings, although no significant change in percentages of serum components was observed between the original and final analyses.

Anaplasma Ovis in the United States

Experimental Studies with Sheep and Goats

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THE RECOVERY of a species of Anaplasma from a flock of sheep in Kansas has been reported recently.[1] Individuals of this flock originated from the states of Colorado, Wyoming, and Montana. Relatively mild Anaplasma infections were produced following experimental inoculation of blood containing Anaplasma into nonsplenectomized sheep, while acute anaplasmosis developed in a splenectomized lamb. All infected animals developed positive reactions to the anaplasmosis complement-fixation (c.f.) test.

This report concerns further studies conducted to determine the identity of the species of Anaplasma observed. The results obtained in experimental transmission have led to the conclusion that the organism is identical to *Anaplasma ovis*.[2]

LITERATURE

Reports of anaplasmosis in sheep have appeared in the literature since 1912. Bevan,[3] Schellhase,[4, 5] Trautmann,[6] and Domizio[7] described outbreaks of disease attributed to an Anaplasma in sheep and goats in various sections of Africa. De Kock and Quinlan[8] consider the report of Domizio in 1919 to be the first authentic description of anaplasmosis in animals other than cattle. Sergent *et al.*[9] reported the occurrence of anaplasmosis in Algerian sheep which was not transmissible to cattle. Further studies were reported by Lestoquard[9] who described and differentiated the organism, *A. ovis*, from Anaplasma of cattle. An anemia varying from mild to severe was the predominating symptom produced in sheep and goats. Necropsy lesions were similar to bovine anaplasmosis. Naturally infected carriers were found in sheep and goats in Algeria.

De Kock and Quinlan[8, 10, 11] and De Kock[12, 13] observed anaplasmosis in South African sheep during observations on the effects of splenectomy. In a study of 32 nonsplenectomized sheep inoculated with *A. ovis*, no mortality was observed. Anemia varying from mild to severe was produced, however. Cattle could not be infected with the ovine Anaplasma, nor did it immunize them against *Anaplasma marginale*. Splenectomy increased the susceptibility of sheep and goats to the infection and caused relapses in carriers. Lestoquard *et al.*[14] also observed recidivations of *A. ovis* following splenectomy.

A number of reports followed concerning observations of anaplasmosis in sheep and goats in Africa,[15-16] France,[17-20] Italy,[21] Sardinia,[22] Russia,[23-24] Palestine,[27] Turkey,[25] and Indochina.[26] Cuille *et al.*[20] demonstrated by inoculation studies that the ovine Anaplasma of Algerian sheep was identical with that observed in the sheep of France. Dschunkowsky[28] described a pear-shaped Anaplasma-like structure in the blood of sheep in Yugoslavia. He classified the organism in a new genus, Pirochroma.

In reference to the pathogenicity of *A. ovis* as seen in the field, DeKock and Quinlan[11] described the organism as causing a mild enzootic blood disease. Du Toit[29] stated that in North Africa the disease appeared to be more severe than in South Africa, where acute cases had not been diagnosed. After a study of the disease in France, Cuille[20] concluded that acute outbreaks were the result of lowered resistance brought about by other infections. Gilbert[27] observed large numbers of goats and sheep affected with acute anaplasmosis in Palestine. The disease was more prevalent after seasons in which forage was scarce and the animals in poor condition.

Neitz[30] and Enigk[31] found that *A. ovis* was transmissible to the blesbok and eland antelope. Neitz[32] summarized the various characteristics of *A. marginale*, *Anaplasma centrale*, and *A. ovis* in ruminants. In studies on the insect transmission of *A. ovis*, the following tick vectors have been incriminated: *Ornithodorus lahorensis*,[25] *Dermacentor silvarum*,[26] *Rhipicephalus bursa*,[27] and *Rhipicephalus turanicus*.[28]

In Louisiana, Rees[29] reported the failure of 4 splenectomized sheep to relapse with *A. ovis*. Recently, Jensen[47] reported on a case of suspected anaplasmosis in sheep in Utah. Marginal bodies were observed in the erythrocytes of animals showing an acute anemia.

PROCEDURE

Two lambs, which had received blood inoculations from sheep in the field as previously described,[1] served as the original source of Anaplasma. Further sources included sheep and goats through which the organism was passed during the course of study. Transmission was accomplished with 5 ml. of citrated blood injected intravenously. Local sheep and goats of mixed breeds were used in the inoculation studies. The ages of these ani-

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mals varied from 3 weeks to 5 years, 8 sheep and 4 goats being over 1 year of age, and 9 sheep and 4 goats less than a year old at the time of inoculation. In early studies,[1] the Anaplasma infection was complicated with *Eperythrozoon ovis*. Subsequently, the latter organism failed to appear following inoculation of a sheep, and this uncomplicated strain of Anaplasma was used in further studies.

Data gathered included body temperature, total erythrocyte and leukocyte counts, hemoglobin determinations, hematocrit values, and observations on Giemsa-stained blood smears. Percentages of Anaplasma-infected erythrocytes were determined from a count of 1,000 red blood cells. Temperature and hematologic data were gathered on alternate days during the prepatent period and daily during the period of Anaplasma patency.

Blood serums from the experimental animals were subjected to the anaplasmosis c.f. test on alternate days during the prepatent period, and irregularly thereafter. *Anaplasma marginale* antigen was used routinely in these tests and was prepared by the method of Price *et al.*[6] *Anaplasma ovis* antigen was prepared in the same manner using goat blood that contained Anaplasma infecting 41.4 per cent of the erythrocytes. The c.f. test procedure and interpretation followed that of Price *et al.*[6]

Twelve splenectomized calves were used to determine the infectivity of the ovine Anaplasma for cattle. Each was inoculated intravenously with 5 ml. of citrated sheep blood. Five of these calves received blood containing an Anaplasma erythrocytic infection of 1 per cent, and 7 were inoculated with blood containing a 12 per cent infection. Blood smears were examined microscopically for Anaplasma at three-day intervals, and the serums were tested weekly for Anaplasma c.f. antibodies. The calves were later challenged with a known source of *A. marginale*.

To determine the effects of splenectomy on the disease, 5 sheep were splenectomized prior to inoculation with *A. ovis*. Three sheep and 3 goats were splenectomized while they were in the carrier state of the disease.

RESULTS

All sheep and goats which were inoculated with sheep or goat blood containing Anaplasma developed infection characteristic of *A. ovis*.[2,5] As a rule, the disease was mild with its effects being demonstrated subclinically. Anaplasma appeared in the blood of sheep and goats on an average of 12 days after inoculation, and increased gradually over an additional average period of nine days until maximum erythrocytic infection was reached. Blood values began to decline as the Anaplasma increased in numbers, and the lowest values were observed after the organism had reduced from maximum numbers. In nearly all animals, evidence of anemia was observed in blood smears and included anisocytosis, polychromatophilia, and punctate basophilia. These blood regenerative changes occurred during the period of decline in the numbers of Anaplasma.

In nonsplenectomized animals, 14 sheep and 11 goats, the parasite showed greater pathogenicity for goats (table 1). An average maximum erythrocytic infection of 2.0 per cent occurred in goats compared with 0.8 per cent in sheep. Average red cell counts reduced from 16.38 to 7.93 million cells per cubic millimeter in goats, a reduction of 8.5 million cells. In sheep, counts reduced from an average of 10.57 to 6.32 million cells per cubic millimeter, a reduction of 4.3 million cells. Correspondingly greater reductions were also found in the hemoglobin and hematocrit values of goats. Total leukocyte counts usu-

TABLE 1—Anaplasma Ovis Infection in Experimentally Inoculated Sheep and Goats

Days from inoculation to:			Maximum r.b.c. infection (%)	Average prepatent values			Lowest values observed during infection		
Pos. smear	Maximum Anaplasma infection	Lowest r.b.c. count		r.b.c. 10^6/cu. mm.	Hematocrit %	Hb. gm./100 cc.	r.b.c. 10^6/cu. mm.	Hematocrit %	Hb. gm./100 cc.
DATA (RANGE) OF 14 NONSPLENECTOMIZED SHEEP									
5–18 (12)*	14–25 (19.8)	20–35 (27)	0.1–3.2 (0.8)	8.60–12.50 (10.57)	29–41 (35.5)	10.4–14.5 (11.8)	3.87–8.14 (6.32)	19–34 (24.4)	5.3–11.5 (8.0)
DATA (RANGE) OF 11 NONSPLENECTOMIZED GOATS									
8–23 (14.5)	15–30 (23)	23–34 (28.4)	0.6–6.8 (2.0)	13.12–20.37 (16.38)	29–45 (35.2)	10.5–14.6 (12.2)	3.25–12.84 (7.93)	10–24 (16.6)	3.8–8.9 (6.2)
DATA (RANGE) OF 7 SPLENECTOMIZED SHEEP									
5–16 (11.4)	14–31 (21.6)	23–35 (27)	4.6–39.4 (17.8)	8.00–11.00 (9.48)	29–38 (33.5)	11.3–12.4 (11.9)	1.95–5.71 (3.90)	7–20 (12.8)	2.8–7.3 (4.6)
DATA (RANGE) OF 3 SPLENECTOMIZED GOATS									
10–15 (12)	16–22 (19.6)	21–27 (24)	2.0–41.4 (15.5)	12.32–13.85 (13.17)	30–33 (31.7)	10.0–11.8 (11.1)	1.40–7.40 (4.75)	6–17 (13)	2.6–6.5 (5.0)

* Number in parentheses is the mean.

ally remained unchanged. Body temperatures were usually unchanged, although readings as high as 105.8 F. were observed in several animals at the peak of Anaplasma infection.

No clinical evidence of acute disease was observed in these animals, although in some the mucous membranes showed marked evidence of anemia. One goat, whose erythrocyte count had dropped from 15.25 to 3.25 million cells per cubic millimeter, showed reduced activity for several days during the period of acute anemia, but the appetite was unaffected. No marked variation in age susceptibility was noticed in the animals studied; however, the greatest reductions in blood values occurred in the older animals.

As reported previously,[1] the morphology of the parasite was identical to *A. marginale* (fig. 1, 2). An examination was made of 1,000 Anaplasma bodies to determine their relative positions in the erythrocyte. Sixty per cent were situated marginally, and 40 per cent were submarginal or central in location. This is comparable with previous reports[26, 33] indicating that about two-thirds of the forms of *A. ovis* were marginal. *Anaplasma marginale* was reported as having 90 per cent marginal forms.[33]

Splenectomy of sheep and goats increased considerably their susceptibility to the Anaplasma infection (table 1). Removal of the spleen of known carriers resulted in a relapse of the disease. Acute anaplasmosis with clinical symptoms occurred in 3 splenectomized sheep and 1 goat. Comparatively milder attacks occurred in 7 other animals. Symptoms included anorexia, dyspnea on exertion, and generalized weakness. Temperatures were elevated to a maximum of 107.4 F. during the early clinical symptoms. Necropsy lesions of 1 sheep which died included anemia, slight icterus, and a nutmeg-colored liver showing evidence of passive congestion.

All sheep and goats inoculated developed positive reactions to the anaplasmosis c.f. test. Positive reactions occurred eight days before the microscopic appearance of bodies in the blood to seven days afterward, the average time being two days prior to the Anaplasma patency. In a quantitative comparison of the Anaplasma c.f. antibodies by end-point titrations of serums,[42] sheep and goats infected with *A. ovis* developed less antibody at various stages of the disease than cattle harboring *A. marginale*. In both diseases, the greatest quantity of c.f. antibody was present during the patent phase of the disease, and the least during the carrier state. The majority of sheep and goats which were carriers of *A. ovis* continued to react positively one year after inoculation; however, some known carriers gave negative reactions in serum dilutions as low as 1.0:2.5 against either *A. marginale* or *A. ovis* antigens. A cross

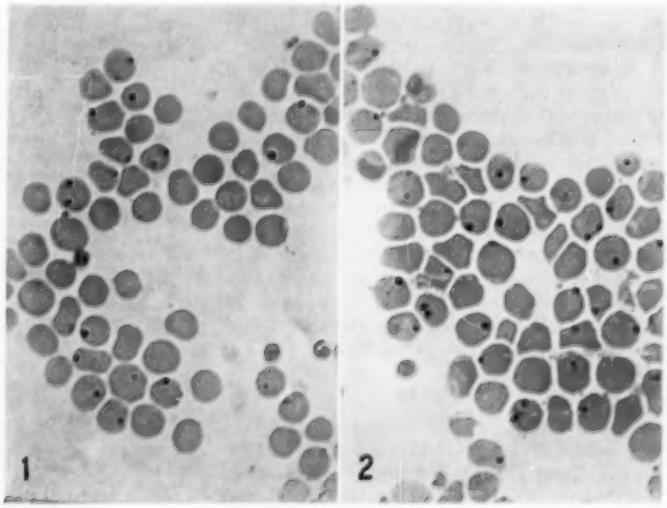

Fig. 1 — Anaplasma ovis recovered from sheep blood.

Fig. 2 — Anaplasma ovis recovered from goat blood.

antigenicity was observed between *A. ovis* and *A. marginale* in the c.f. test. Identical reactions were obtained in 50 positive and negative goat, sheep, and cattle serums tested with both antigens.

The experimental attempt to transmit the ovine Anaplasma from sheep to splenectomized calves was unsuccessful. All calves remained normal with negative blood smears for 80 to 120 days. Complement-fixation tests of the calves' serums also remained negative during this time. The calves then were found to be fully susceptible to infection with *A. marginale*.

In an attempt to pass *A. ovis* through cattle, a splenectomized calf was injected with infective sheep blood. Sixteen days later, blood was drawn from the calf and inoculated into splenectomized sheep. *Anaplasma ovis* was not recovered indicating that the organism had not survived in the calf.

DISCUSSION

The presence of an Anaplasma in sheep transmissible to goats but not to cattle indicates that the organism is identical to *A. ovis*.[2] No information is available at present on the origin or distribution of the organism in this country. Considering the origin of the sheep from which the parasite was first recovered in Kansas, it seems probable that the disease occurs in sheep in the Rocky Mountain area. The generally mild nature of the condition in all probability has precluded its earlier observation. It remains to be determined if *A. ovis* occurs naturally in goats in the United States.

Although *A. ovis* usually produces a subclinical infection, it is evident from the literature that sporadic outbreaks of the acute disease may occur. Conditions of heavy parasitism, malnutrition, debilitating diseases, and increased individual susceptibility may predispose to the acute disease. It is apparent that the possibility of ovine anaplasmosis should be considered in cases of anemia occurring in sheep and goats in this country. Diagnosis may be confirmed by blood smear examination and by the anaplasmosis complement-fixation test.

SUMMARY

1) An anaplasma identified as *Anaplasma ovis* was recovered from sheep originating from the Rocky Mountain area of the United States. The organism produced variable degrees of subclinical anemia in sheep and goats. Cattle could not be infected with the parasite, nor did it produce any detectable immunity in these animals against *Anaplasma marginale*.

2) Experimentally, the strain of *A. ovis* studied showed greater pathogenicity for goats than for sheep. An average reduction of 8.5 million erythrocytes per cubic millimeter of blood occurred in goats compared with an average reduction of 4.3 million cells in sheep. Splenectomy increased the susceptibility of both.

3) A cross antigenicity was observed between *A. ovis* and *A. marginale* in the anaplasmosis complement-fixation test.

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Clostridium Perfringens Type B from Enterotoxemia in Young Ruminants

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Clostridium perfringens type B has been shown to be the cause of lamb dysentery in England by Gaiger and Dalling.[1]

In 1933, Tunnicliff[2] described a disease in Montana which was apparently the same, although his organism did not liquefy coagulated serum nor clot alkaline egg medium. Griner and Bracken[3] reported finding *Cl. perfringens* type C in acute hemorrhagic enterotoxemia of calves and Griner and Johnson[4] found the same organism in lambs. Newsom[5] describes enterotoxemia due to *Cl. perfringens* type D as a frequent malady of nursing lambs 4 to 12 weeks of age. Recovery of *Cl. perfringens* type B has not been described in the United States.

Clostridium perfringens type B produces two major toxins,[6] the *beta* toxin and the *epsilon* toxin. The *beta* toxin is also the major toxin of *Cl. perfringens* type C while the *epsilon* toxin is the major toxin of *Cl. perfringens* type D. Montgomerie and Rowlands[7] showed that by suitable cultural methods the type B organism could be made to produce almost pure *beta* or pure *epsilon* toxin. This relationship between the type of medium and the type of toxin formed *in vitro* is also apparent *in vivo* in lambs affected with lamb dysentery. English workers[6] found that in the intestinal tract of lambs infected with *Clostridium welchii (perfringens)* type B, the toxin formed depended on the age of the lamb and upon the food ingested. *Beta* toxin alone has frequently been demonstrated postmortem in the bowel contents of lambs dying within a few hours or a few days of birth and *epsilon* toxin alone in those lambs dying when 10 to 14 days old. Montgomerie[7] was not able to demonstrate both toxins simultaneously in the intestinal content of lambs infected with *Cl. perfringens* type B.

The capacity to produce toxin is readily lost by many strains.[8] A type B strain used by Glenny[10] for immunizing horses lost its ability to produce the *epsilon* toxin. It was then indistinguishable from the type C organism.

Materials and Methods

In routine diagnostic work at this laboratory, the supernatant fluid of centrifuged intestinal contents from ruminants suspected of having enterotoxemia was routinely tested for toxicity by intravenous inoculation of mice. Toxic samples were then tested for toxins of *Cl. perfringens* by serum neutralization tests employing serums (*Cl. welchii* [*perfringens*], types A, B, C, D, and Bosworth) secured from Wellcome Research Laboratories. Each of the five known types was identified by recognizing the toxin or combinations of toxins produced as shown in table 1, which was supplied with the serum.

The following procedure was used to determine the types of toxin in the intestinal contents:

1) Mice, previously heated under a lamp or near a heater to dilate the lateral veins of the tail, were injected intravenously with graded amounts of supernatant fluid of centrifuged intestinal contents from the animals suspected of having enterotoxemia. The maximum amount injected was usually 0.25 cc. All mice which suffered shock as evidenced by an immediate reaction were discarded from the test. The amount injected was increased or decreased to have death occur in from five minutes to 24 hours postinjection.

2) If this test indicated the presence of toxin, neutralization tests were performed by mixing supernatant fluid with equal volumes of diluted antiserum (1 part antiserum to 9 parts distilled H_2O) of each of the *Cl. perfringens* types. The mixtures were allowed to stand at room temperature for 30 minutes. Three mice were then injected with each of the toxin-antitoxin mixtures. These mice were then observed for two days and the results recorded and interpreted (table 1).

Cultures of *Cl. perfringens* were established by picking single colonies from blood agar plates which had been streaked with the toxic bowel contents and incubated anaerobically. These cultures were then tentatively identified as *Cl. perfringens* on the basis of the following criteria: colony morphology, hemolysis, cell morphology, and stormy fermentation of milk. Cultures were then placed in stock for typing.

Toxin was produced *in vitro* in a liver-infusion dextrose broth. Supernatant fluid from 18-hour, eight-hour, and five-hour cultures were typed for

TABLE 1—Typing of Clostridium Welchii

Neutralized by serum of type	Not neutralized by serum of type	Interpretation	
		Toxin	Type
A	.....	Alpha	A
B	A, C, and D	Beta and Epsilon	B
C	A and D	Beta	C
D	A and C	Epsilon	D
Bosworth	A, B, C, and D	Lambda	E

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toxin by the same method as that used for intestinal contents. However, if the cultures were nontoxic or if neutralization tests were negative for the *epsilon* toxin, it was necessary to activate the supernatant fluid by treating with trypsin and repeating the test using this trypsin-treated supernatant fluid. (*Epsilon* toxin is produced by the organism in the form of a prototoxin which gradually changes to toxin in the test tube. This conversion from prototoxin to toxin is known to be hastened by the presence of trypsin. Where *epsilon* toxin is present in cultures prior to treatment with trypsin, the toxicity of the sample is enhanced by trypsin treatment.) It was not necessary to treat supernatant fluids from intestinal contents with trypsin since trypsin is known to be present in the small intestine. This trypsin treatment was accomplished by adding 0.25 per cent of trypsin (Difco 1:250) to the supernatant fluid and incubating at 37 C. in a water bath for 45 minutes.

RESULTS

Two lambs from a small purebred flock which was experiencing losses were presented for necropsy and diagnosis. Their exact age was not known but all lambs in the flock were between 1 and 4 weeks old. Anaerobic blood agar plates of intestinal contents of both lambs yielded nearly pure cultures of *Cl. perfringens*. The supernatant fluid of intestinal contents of each was toxic for mice, but from neutralization tests it was found that one (24066) contained the *beta* toxin while the second (24099) contained the *epsilon* toxin. Cultures were established from both for *in vitro* toxin production. Both of these cultures consistently produced the *epsilon* toxin only (table 2).

Supernatant fluid of intestinal contents of a lamb from a second flock of approximately 50 head was toxic. Six lambs from this flock had died in one week with a history suggesting enterotoxemia. Blood agar plates streaked with toxic intestinal material and incubated anaerobically yielded nearly pure *Cl. perfringens*. Neutralization tests showed the presence of the *beta* toxin only. Cultures from this lamb (24438-1, 2, and 3) consistently yielded only the *epsilon* toxin except, in one instance, when an eight-hour culture was neutralized only by the type B antitoxin, indicating the presence of both the *beta* toxin and the *epsilon* toxin.

A 3-day-old Aberdeen Angus calf (24317) showed hemorrhagic enteritis on necropsy. Intestinal contents were toxic for mice and yielded nearly pure *Cl. perfringens* on anaerobic plates. The toxin present was neutralized only by the type B antiserum, indicating the presence of both *beta* and *epsilon* toxins. *In vitro*, the cultures established did not produce demonstrable toxin.

DISCUSSION

In a small flock, where the lambs were 1 to 4 weeks old, 2 died of enterotoxemia about seven days apart, with several other deaths intervening. In the first lamb, enterotoxemia due to *Cl. perfringens* type C was diagnosed on the basis of finding the *beta* toxin alone in the bowel content, while in the second lamb type D enterotoxemia was diagnosed when the *epsilon* toxin alone was found to be present. A *Cl. perfringens* culture isolated from toxic intestinal content of the lamb dead of an apparent type C intoxication (*beta* toxin) was shown to have the potential to produce the *epsilon* toxin *in vitro*.

Intestinal material from a single lamb from another flock of 50, in which six deaths occurred in one week, was found to contain the *beta* toxin alone. *In vitro*, three cultures established from the toxic intestinal material all consistently produced the *epsilon* toxin except one (24438-2)

TABLE 2—Results of Toxin Neutralization Tests with Intestinal Contents and Corresponding Cultures

Source	Type of toxin in intestinal contents	*Cl. perfringens* type indicated	Type of toxin *in vitro* — Hours of incubation 18	8	5	*Cl. perfringens* type indicated
Lamb 24066	Beta	C	Epsilon *	Epsilon *	Nontoxic	D
Lamb 24099	Epsilon	D	Epsilon *	Epsilon *	Epsilon	D
Lamb 24438-1	Beta	C	Epsilon *	Epsilon	Epsilon	D
24438-2	Beta	C	Epsilon *	Beta and epsilon	Epsilon	D or B
24438-3	Beta	C	Epsilon	Epsilon	Epsilon	D
Calf 24317 (1, 2, 3)	Epsilon and beta	B	Nontoxic	Nontoxic	Nontoxic	—

* Trypsin-treated; *epsilon* toxin present both before and after treatment; toxicity was enhanced by treatment.

which produced both the *beta* and the *epsilon* toxin on one occasion.

Intestinal contents from a 3-day-old calf were found to contain both the *beta* and the *epsilon* toxin, indicating the presence of *Cl. perfringens* type B. Cultures established from this material were consistently nontoxic *in vitro*.

Failure to produce the *beta* toxin *in vitro* (except in one instance) can probably be attributed to the medium used for toxin production, since it has been shown by other workers that the type B organism could be caused to produce one or the other toxin alone by varying the cultural medium. The finding of one toxin alone in the bowel content (except 24317) is not inconsistent with the findings of English workers with *Cl. perfringens* type B.

All of the cultures had been held in stock for some time prior to demonstration of toxin production *in vitro*. Inability to confirm toxin production in cultures of 24317 may have been due to long storage since other workers have found that some strains rapidly lose the ability to produce toxin.

The significance of this work lies in the fact that the routine laboratory diagnosis of enterotoxemia is performed on supernatant fluid of intestinal contents from animals suspected of having enterotoxemia by methods similar to those described in this paper. If the results of such tests show that the *beta* toxin alone is detectable, the diagnosis would be commonly reported as *Cl. perfringens* type C enterotoxemia and where the *epsilon* toxin alone is detectable, the diagnosis would be commonly reported as *Cl. perfringens* type D enterotoxemia. However, in Montgomerie's [7] work with *Cl. perfringens* type B from cases of lamb dysentery, he clearly states that he was never able to demonstrate the presence of both the *beta* and *epsilon* toxin in a single sample of intestinal contents. However, he was able to prove the causative organism to be *Cl. perfringens* type B. Similar results were obtained by this author. It must be assumed, therefore, that where the *beta* toxin is demonstrated in intestinal contents, the causative organism may be either *Cl. perfringens* type B or *Cl. perfringens* type C. Similarly, where the *epsilon* toxin is demonstrated to be present in intestinal contents, it must be assumed that the causative organism is either *Cl. perfringens* type B or *Cl. perfringens* type D. This information should be taken into account in the interpretation of such tests.

SUMMARY

1) Evidence has been presented indicating the presence of enterotoxemia in lambs and in calves in the United States due to *Clostridium perfringens* type B.

2) *Clostridium perfringens* cultures from apparent cases of *Cl. perfringens* type C have been shown to have the ability to produce the *epsilon* toxin (major toxin of type D).

3) Lambs with enterotoxemia due to the *beta* toxin alone (apparent type C) and the *epsilon* toxin alone (apparent type D) have been found in the same disease outbreak in a single flock.

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Studies on the Etiological Agent of Epididymitis in Rams

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SIMMONS AND HALL,[1] in a report on epididymitis in rams, described the etiological agent as a Brucella-like organism. Their classification was based on morphology, atmospheric requirements, growth characteristics, biochemical reactions, serology, and dye and antibiotic sensitivity.

Buddle and Boyes[2] in 1953 isolated an organism from the semen and lesions in the genitalia of rams from various districts in New Zealand. They also recovered an identical organism from diseased fetal membranes, aborted lambs, and the colostrum of naturally infected ewes. A disease indistinguishable from the naturally occurring condition was induced experimentally by inoculating the organism into susceptible sheep. These investigators described the bacterium as possessing characteristics of the genus Brucella. Later publications by Hartley, Jebson, and McFarlane[3] referred to the condition as ovine brucellosis.

Epididymitis in rams has been reported by McGowan and Shultz[4] in California. Through the courtesy of W. T. K. Hall and M. B. Buddle, we have received cultures isolated from animals in Australia and New Zealand. From a critical examination, the organisms are apparently identical with those isolated in California.

The isolation of this organism from widely separated areas and the suggestion that it be placed in the genus Brucella has stimulated investigations with the objective of classifying this organism.

While possessing certain characteristics common to Brucella, the organism also possesses a number of characteristics that would place it in another genus.

The purpose of this paper, therefore, is to report the results of a critical examination of the organism and to present data that would suggest placing it in a genus other than Brucella.

METHODS

In addition to serological comparisons, the cultures were compared with Brucella with respect to their metabolic activity as resting cells, H_2S production, and urease activity.

The cultures used for manometric study were a California strain (391) isolated from the affected epididymis of a ram and Australian culture, Q 4. Both grew well without added CO_2.

The organisms were prepared for manometry by inoculating Blake bottles containing tryptose agar enriched with 5 per cent bovine blood. After 48 hours of incubation, the cells were washed off the mediums with saline, filtered through a small Buchner filter, and washed in Sorenson's M/15 phosphate buffer with the desired pH. They were then resuspended in buffer and used immediately.

Cellular nitrogen was determined by a micro-Kjeldahl technique.

All substrates were prepared at a concentration of 10 mg. per milliliter in buffer. When necessary, the pH was adjusted by the addition of NaOH.

The Warburg vessel contained 1.4 ml. of buffer with the appropriate pH; 0.5 ml. of substrate (5 mg.); 1.0 ml. of cell suspension; and 0.1 ml. of KOH in the center well. Conventional Warburg manometric techniques were employed to determine oxygen uptake.[5] Endogenous rates of oxygen uptake were measured for each experiment and exogenous respiration rates were determined in duplicate. This procedure was then repeated on different harvestings of the cells.

The comparative metabolic activity of the resting cells was determined by using substrates of amino acids, carbohydrates, and compounds that are intermediates in the Krebs tricarboxylic acid cycle. The following amino acids were used as substrates: D, L, and *beta* alanine, L-arginine, D and L-asparagine, D and L-aspartic acid, DL-citrulline, D and L-glutamic acid, glycine, D and L-histidine, L-leucine, L-lysine, DL-ornithine, DL-phenylalanine, L-proline, L-serine, D and L-threonine, D and L-valine, and urea.

The following carbohydrates were used as substrates: adonitol, arabinose, cellobiose, dulcitol, fructose, galactose, glucose, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose.

RESULTS

The Comparative Metabolic Activity.—The results of this phase of the investigations are shown in table 1. Both organisms

TABLE 1—Comparative QO$_2$N Values

Substrate	*Br. melitensis*	Ovine organism
D-aspartic	0	100
Citrulline	75	0
Proline	95	0
Serine	0	175
D-alanine	117	150
L-alanine	32	54
L-glutamic acid	85	150
Glucose	235	0
Fructose	130	0
Galactose	75	0
Xylose	90	0

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use all the compounds in the tricarboxylic acid cycle with similar uptake rates and neither organism is active on any of the remaining substrates.

Hydrogen sulfide was not produced and nitrates were not reduced by the ovine organisms.

Serology.—A total of 41 blood samples from rams from field cases of epididymitis and six from experimental rams were tested for agglutinins against Brucella. All were negative with 19 *Brucella abortus* antigen and an antigen similarly prepared from *Brucella melitensis*. An antigen for the agglutination test was also prepared from the California ovine organism. Because of the slimy nature of the growth, it was necessary to filter and wash the suspension. It was resuspended in phosphate buffer at pH 6. This antigen was not stable for more than 48 hours. In 38 tests, there was agreement in 22, 18 of these being both clinically and serologically positive. The antigen was not agglutinated by positive Brucella serum.

DISCUSSION

The isolation of the genus Brucella from sheep has been reported from various countries, particularly continental Europe. In most cases, the organism was identified as *Br. melitensis*. The identification of the genus was generally confirmed by serological techniques. A partial review of the literature on Brucella infection in sheep has been made by Stoenner,[6] who also recorded the isolation of *Br. abortus* from 1 ewe during the course of an epidemiological investigation of Q-fever in southern California. As far as can be determined, this is the only recorded isolation of Brucella from sheep in the United States.

The comparisons herein reported would lead to a conclusion that the organism causing epididymitis in rams should not be considered a member of the genus Brucella. In addition to the lack of any serological relationship, there are several criteria that would justify placing it in another genus.

Amino Acids.—The pattern of amino acid metabolism displayed by the ovine organism indicates that its enzymatic constitution is distinctly different from that of the genus Brucella. The most outstanding difference occurs in the inability of the ovine organism to oxidize those amino acids that are intermediates in the urea cycle, namely, citrulline, ornithine, arginine, and proline. In each species of the genus Brucella there is an active urea cycle, and each species is capable of initiating the functioning of the cycle from one or more of the intermediates.[7,8] Lack of the urea cycle, which is an integral part of both the catabolic and anabolic metabolism of Brucella,[9] strongly indicates that the ovine organism is not a member of the genus Brucella.

The other metabolic differences observed in the ovine organism are its utilization of serine and D-aspartic acid. Members of the genus Brucella do not oxidize these two amino acids.[8,10]

Each species of Brucella contains the enzyme urease and is thereby able to hydrolyze urea. There is abundant evidence to indicate that this is a stable characteristic of the genus.[11-15] The combined number of cultures studied by these authors was 5,515, none of which lacked urease. Pickett and Nelson [16] recently recorded 16 species-intermediate strains as being urease negative. However, by a personal communication, it was explained that these cultures were only quantitatively negative, and that none of his 300 strains was completely negative. The weight of evidence is in opposition to classifying an organism as a member of the genus Brucella if it is totally lacking in urease.

Carbohydrates.—The carbohydrate metabolism of the ovine organisms is also distinctly different from that of the genus Brucella. As early as 1928, McAlpine and Slanetz,[17] while studying 65 cultures with each species represented, observed that they were all glucose utilizers. More recently, Pickett and Nelson [16] examined 102 typical and atypical strains of Brucella and reported that all of these cultures showed a uniformity of fermentive activity on several carbohydrates. These authors used glucose as a routine positive control. McCullough and Beale [18] examined cultures of each species for both the fermentive activity in mediums and the oxidative ability under manometric conditions. Glucose was used consistently under both conditions. That the ovine organism is a species of Brucella that fails to utilize glucose seems improbable.

Buddle and Boyes [2] suggested that the ovine organism was a mutant of *Br. meli-*

tensis. Van Drimmelen [19] reported the isolation of Brucella from sheep and classified it as an atypical strain of *Br. melitensis*. Although atypical in some of its biochemical activities, this strain, according to Cruickshank,[20] was agglutinated by monospecific anti-*Brucella melitensis* serum.

Since the ovine organism did not utilize amino acids in a metabolic pattern similar to Brucella, failed to utilize glucose, lacked the urease enzyme, and displayed no serological relationship, it is suggested that it is not properly classified in the genus Brucella.

The question then arises as to the genus in which the ovine organism should be placed. It possesses many characteristics of the nonpathogenic Neisseria, and this genus was considered. The features in common with Neisseria are as follows: failure as resting cells to utilize carbohydrates, lack of H_2S production, and failure to reduce nitrates. However, the ovine organism is oxidase negative while Neisseria is positive. Brucella is definitely a homogenous group, while Neisseria is composed of organisms possessing a wide variety of characteristics.

It would appear, therefore, that if it is to be placed in an existing genus, consideration should be given to classifying the ovine organism in the genus Neisseria rather than Brucella.

SUMMARY

Comparative examinations were made with *Brucella melitensis* and the organisms isolated from epididymitis of rams in Australia, New Zealand, and California. The organisms isolated from rams in all three areas were identical. A critical comparison, however, indicated that the ovine organism should not be placed in the genus Brucella. It was suggested that consideration be given to classifying it in the genus Neisseria.

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Effects of Dried Preserved Rumen Inoculum on the Rumen Microorganisms of Lambs

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MUCH has been written in recent years concerning the merits of various rumen stimulants* as profloran, ru-bac, bovinoc, rumenade with culture nutrients, ru-vitazyme concentrate, ru-zyme, rumen stimulant, bovine ruminatoric, and floravite. The use of these products has varied from simple stimulation of the rumen wall to the correction of rumen dysfunction brought about by disease.

Chemicals, enzymes, normal rumen contents, commercially prepared rumen inoculums, and combinations of these have been used. More recent investigation has been directed at the establishment of normal flora and fauna in young animals and the re-establishment of flora in older animals.

Ruminants are anatomically and physiologically equipped with a digestive system primarily adapted for utilization of large quantities of roughage. The investigation herein reported deals with factors affecting the utilization of roughage by sheep.

REVIEW OF THE LITERATURE

A balance of rumen microorganisms as to kind and number has been stressed as the basis for proper function. Bryant and Burkey[4] found the number of bacteria in the rumen of cows was about the same, regardless of ration, except for an increase in number on a concentrate ration; however, the flora became more complex when alfalfa hay was fed. Burroughs *et al.*,[6] Gall *et al.*,[12] and Hungate *et al.*[21] have reported variation in kind and number of these organisms in ruminants on different rations.

Burroughs *et al.*[6,7] found a decrease of organisms in dry-matter digestion in mixed rations as compared with rations of higher roughage content. Phosphorus and iron were effective in stimulating cellulose digestion by rumen microorganisms.[6]

The work of Pounden *et al.*[24,26-28] encouraged the use of fresh rumen material, especially in young calves. They described a method of collecting rumen material (cud) from a normal cow and reported that Protozoa and certain bacteria associated with hay ingestion were readily established in the rumens of calves on pasture by inoculation with cud material.[27] Similar results were obtained on a ration of alfalfa hay and limited grain.[28] The most noticeable advantage to calves from such inoculations has been in a better quality of coat and less "pot belly" as compared with calves left to nature for establishment of rumen microorganisms. Hibbs *et al.*[19] and Goodwin *et al.*[13] reported the same advantages from using cud material in starting calves on high roughage rations. However, calves inoculated with rumen fluid each week from 2 to 6 weeks of age showed no increase in roughage consumption and weight gain over uninoculated calves.

Attempts have been made to increase the rate of cellulose digestion in ruminants. Using a manometric procedure, McBee[22] was able to show increase in the rate of fermentation of both cellulose and hemicellulose by the rumen fluid of sheep by addition of cellulose to a hay diet. The fermentation of hemicellulose was increased when hemicellulose was added, but the fermentation of cellulose was not increased. McBee found that the number (20 to 30 billion/ml. of fluid) and type (small single rods and cocci in pairs and small clumps) of bacteria remained the same. Head[16] has suggested that the addition of maize or potato starch to a ration of hay depressed the cellulose digestion proportional to the amount of starch added. Addition of nitrogen-rich feed did not counteract this tendency. Heald[17] demonstrated that considerable quantities of xylose-containing constituents of the diet were fermented in the rumen of sheep.

Burroughs *et al.*[6] and Arias *et al.*[1] described so-called "artificial rumen" techniques for studying rumen function. Many techniques for culturing and isolating rumen bacteria have been described.[5,10,11,20]

Some modern therapeutic agents have been found to be detrimental not only to harmful microorganisms of the digestive system but also to those commonly considered to be of benefit. Heald and Oxford[18] found that streptomycin in relatively high concentrations would destroy certain of the

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* Profloran (Jensen-Salsbery Laboratories); ru-bac (Haver-Glover Laboratories); bovinoc (Fort Dodge Laboratories); rumenade (Norden Laboratories); ru-vitazyme (Armour Veterinary Laboratories); ru-zyme (Rumelk Co.); rumen (Goshen Laboratories); ruminatoric (Curtis Laboratories); floravite (Hance Bros. and White Co.).

rumen microflora while not affecting the microfauna. Morris[20] suggested that antibiotics may destroy bacteria desirable for digestion. Wasserman *et al.*[29] found that chloramphenicol† used *in vitro* adversely affected rumen microorganisms. Turner and Hodgetts[28] reported that aureomycin® hydrochloride (23.6 to 27.1 mg./kg.) caused a 75 per cent drop in ruminal flora within two hours, with reduction in appetite and body weight. The calves responded favorably to the administration of ruminal fluid and yeast extract.

Rumen dysfunction has been attributed to nearly every common disease of the ruminant.[9, 14] Many commercial products have been compounded in an attempt to duplicate the action of fresh rumen material in establishing the normal flora in young ruminants and correcting dysfunctions in older animals.[9, 14, 15, 28]

The investigation reported here was initiated in an attempt to establish the value of one typical commercial product presented as a substitute for fresh rumen material. The three objectives were to determine whether dried preserved rumen culture: (1) is of value in stimulating the activity of the rumen flora in lambs, (2) is of value in restoring the rumen flora in lambs where such has been reduced by drugs, and (3) produces any toxic effects when fed to lambs.

MATERIALS AND METHODS

Sixteen lambs, ewes and castrated males, representing four breeds were used. They were placed in four lots of 4 lambs each. Records of feed consumption and weight gains were kept. Each lamb was individually fed 0.5 lb. of oats and 0.15 lb. of cottonseed meal per day and all the hay (7.74 % crude protein) it would consume at two feedings per day. Steamed bone meal, salt, and water were supplied free choice.

A preliminary feeding period of ten days was used to accustom the lambs to new surroundings and new ration. An additional 42-day period was used to make adjustments in the ration so it would just maintain body weight or cause very slight gains.

Bacterial counts of the rumen contents of each animal were made at the close of the adjustment period. Rumen material was collected by stomach tube. At this time half of the animals (2 lots of 4 lambs each) were fed chloramphenicol at varying rates ranging from 1 to 8 Gm. every other day for three treatments. On the fourth treatment (6th day) the dosages were reversed, the lambs which had been receiving the least dosage receiving the greatest and vice versa. Then each of the 8 lambs received 7 Gm. of chloramphenicol for the two remaining treatments.

Rumen samples were checked for total numbers of organisms on the days alternate to the chloramphenicol treatments. One gram of fresh, solid rumen material was diluted with trypticase soy broth (15 Gm./liter of water). Dried, preserved rumen inoculum was similarly checked. Each dilution was shaken on a mechanical shaker for three minutes. Dilutions were made at 10^7, 10^8, 10^9, 10^{10}, 10^{11} and cultured on trypticase agar (2 % agar) for 72 hours at 37 C. The air was evacuated from the culture containers and replaced with CO_2. Counts were made from dilutions that contained less than 300 colonies per plate.

Administration of dried preserved rumen inoculum was begun immediately after the 11-day treatment period with chloramphenicol. The lambs in lot 4 (treated) and in lot 2 (control) received dried preserved rumen culture daily by capsule, 10 Gm. per lamb per day for 30 days. Two lambs from each of lot 2 and 4 were then slaughtered. Certain animals (table 1) were then placed on 50 Gm. of inoculum per day for seven to 21 days to study only the toxicity of the material. The experiment was completed either by slaughtering the lambs or by discontinuation of treatment.

The 5 lambs which remained at the end of the experiment were placed on a ration of hay only. After a short adjustment period, the pH of rumen contents was taken for four consecutive days. These lambs were again given the dried preserved rumen inoculum at the rate of 10 Gm. per day per lamb. The pH was then determined at two, three, and six days with the Beckman pH Meter.

Cellulose digestion trials were made in an "artificial rumen," comparing the action of dried preserved rumen inoculum with fresh strained rumen fluid and fresh solid material. The artificial rumen used was a modification of that described by Burroughs *et al.*[5] The digestion medium was trypticase agar as described by Bauman[2] containing:

10.0	Gm. trypticase
10.0	Gm. phytone
5.0	Gm. glucose
4.0	Gm. sodium chloride
1.0	Gm. sodium citrate
0.2	Gm. sodium sulfate
0.2	Gm. cysteine hydrochloride
15.0	Gm. agar
0.001	Gm. resazurin
1000.0	cc. distilled water

A total of 25 ml. of digestion medium was placed in a 40-ml. digestion tube. A strip of No. 40 Whatman filter paper 1 by 10 cm. was placed in each tube. One milliliter of rumen fluid was placed in tubes to check for digestive action. One gram of rumen solids or 1 Gm. of dried preserved rumen culture was placed in some tubes. Other tubes were incubated as controls containing only the digestion medium and cellulose. The digestion time was 72 hours.

† Chloromycetin ®

TABLE 1—Amounts of Chloramphenicol and Dried Preserved Rumen Culture Administered

Lot (No.)	Animal (No.)	Sex[a]	Breed	Chloramphenicol treatments (Gm.) For 3 days	For 1 day	For 2 days	Total for 11-day period	Preserved rumen culture treatment 30-days[b] Gm./day	Additional days at 50 Gm./day
1	1	E	Corriedale	....	....	....	....	....	[d]
	2	C	Columbia	....	....	....	....	....	[e]
	3	C	Suffolk	....	....	....	....	....	[e]
	4	C	Rambouillet	....	....	....	....	....	21[e]
2	5	C	Rambouillet	....	....	....	....	10[c]	....
	6	C	Hampshire	....	....	....	....	10[c]	....
	7	E	Hampshire	....	....	....	....	10	21[d]
	8	C	Corriedale	....	....	....	....	10	21[e]
3	9	C	Columbia	1	8	7	25	....	[f]
	10	C	Columbia	2	7	7	27	....	7[f]
	11	E	Suffolk	3	6	7	29	....	21[d]
	12	C	Hampshire	4	5	7	31	....	[f]
4	13	C	Columbia	5	4	7	33	10[c]	....
	14	E	Hampshire	6	3	7	35	10	21[d]
	15	C	Hampshire	7	2	7	37	10[c]	....
	16	E	Hampshire	8	1	7	39	10	21[d]

[a] E ewe; C castrated male; [b] experiment was terminated at end of this period for effects of stimulation of inoculum; however, some animals were carried longer, as indicated, on increased dosages to check for toxicity of the inoculum; [c] animals destroyed after receiving preserved rumen culture for 30 days; [d] animals not destroyed and used for additional pH determinations; [e] animals destroyed after 51 days (additional 21 days); [f] animals destroyed after 37 days (additional 7 days).

RESULTS AND DISCUSSION

The cottonseed meal, included initially, was soon removed from the ration since chemical analysis and rate of gain indicated that it was not needed for purposes of this study. By the end of 42 days, the lambs had reached a relatively static weight (table 2). Rumen samples were collected with difficulty because of the high fiber content of the ration and the relatively low water intake during the winter months. There was a great variability in total bacterial counts when made from the solid portion of the rumen material. Chloramphenicol caused a reduction in total numbers of organisms, but this was difficult to demonstrate due to the action of the drug against the fast-growing types (those which normally spread over the entire medium) of organisms allowing the more discrete organisms to grow to discernible colonies. No attempt was made to identify the organisms involved.

The appetites of the lambs which received more than 2 Gm. of chloramphenicol every other day were reduced markedly. No effect on appetite was noted at the 1-Gm. level. At the 2-Gm. level, only the consumption of hay was reduced. All animals went off feed when placed on high levels of the antibiotic. As a result of the reduced food intake, all of the animals treated with chloramphenicol lost weight rapidly (table 2). The animals not receiv-

TABLE 2—The Average Daily Gains for the Three Periods of the Experiment

Lot	Animals	Initial weight (lb.)	42-Day adjustment period	Average daily gains (lb.) 11-Day period chloramphenicol treatment Untreated	Treated	30-Day period inoculum treatment Untreated	Treated
1 (control)	1 to 4	Range 80 to 146	−0.07 to 0.09	−0.18 to 0.18	.....	0.09 to 0.26	.....
		$\bar{x}$ 117	0.00	−0.07	.....	0.18	.....
		sd. 27.35	0.0663	0.1697	.....	0.0672	.....
2 (control & inoculum)	5 to 8	Range 93 to 139	−0.12 to 0.26	−0.45 to 0.45	.....	.....	−0.29 to 0.03
		$\bar{x}$ 115	0.08	0.00	.....	.....	−0.08
		sd. 24.95	0.1775	0.3674	.....	.....	0.1449
3 (chloramphenicol)	9 to 12	Range 101 to 127	−0.07 to 0.09	.....	−1.54 to −0.64	0.03 to 0.23	.....
		$\bar{x}$ 114	0.03	.....	−1.09	0.14	.....
		sd. 11.87	0.0754	.....	0.4284	0.0830	.....
4 (chloramphenicol & inoculum)	13 to 16	Range 87 to 135	0.02 to 0.19	.....	−1.09 to −0.45	.....	−0.23 to 0.23
		$\bar{x}$ 106	0.08	.....	−0.75	.....	0.02
		sd. 21.93	0.0800	.....	0.2723	.....	0.1962

sd—standard deviation.

ing chloramphenicol remained static in weight. The weight differences of these two groups were significant at the 1 per cent level of the "t" test.

Table 2 shows the gains of each lot during the 30-day period in which inoculum was fed. There was no significant difference in gains in these lots. However, in each case any observed difference was in favor of the controls. This was a strong indication that the inoculum was of no effect in returning malfunctioning rumen flora to normal.

Pathological changes indicating any toxic effects were not found in animals receiving either 10 Gm. or 50 Gm. per day of inoculum. The rumen pH of animals receiving 10 Gm. per day of dried preserved rumen inoculum ranged from 6.65 to 9.00, with no animal consistently high or low. No differences were noticed between controls and treated lambs.

Cellulose digestion trials were made *in vitro* for a 72-hour period. No evidence of cellulose digestion by 1-Gm. samples of dried preserved rumen inoculum was obtained. Considerable digestion was observed when 1 Gm. of fresh rumen solids was used as the inoculum. One milliliter of the fresh rumen fluid caused complete maceration of the cellulose material (No. 40 Whatman filter paper). This compared favorably with the findings of Pounden *et al.*[25]

SUMMARY

Under the conditions of this experiment, it was determined that:

1) The dried preserved rumen inoculum used did not stimulate rumen flora of lambs which had been treated with high levels of chloramphenicol.

2) Dried preserved rumen inoculum showed no *in vitro* cellulose digestion, whereas fresh rumen inoculum caused digestion.

3) There was no evidence of toxicity from feeding dried preserved rumen inoculum.

4) Excessive dosage with chloramphenicol was determinental to certain of the rumen flora and to the appetite of lambs.

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Muscular Dystrophy in Lambs as Related to the Tocopherol Levels in the Plasma and the Milk of Ewes and to Various Feeds

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MUSCULAR DYSTROPHY (white muscle disease) has been associated with vitamin E deficiency in calves and lambs.[3, 5, 6, 14, 31, 32, 35, 39, 41, 42]

It has been reported that both experimentally produced and naturally occurring muscular dystrophy could be prevented and successfully treated with vitamin E.[3–5, 11, 12, 14, 15, 20, 21, 31–33, 35, 41, 42] Muscular dystrophic lesions have been produced with vitamin E-deficient rations in lambs,[6] calves,[5, 21] rabbits,[1, 12, 15, 19, 20] rats,[9–11, 18, 22] guinea pigs,[18, 33] mice,[8, 23] dogs,[2] and chickens.[7] A total blood serum tocopherol level below 70 μg./100 ml. in nursing calves and 150 μg./100 ml. in their dams was associated with muscular dystrophy in calves in Scotland,[32] but in Montana muscular dystrophy did not develop in calves, although it was shown that the tocopherol level in the plasma had remained at nondetectable amounts for two and three weeks.[20]

The supplementation of 150 mg. of *alpha*-tocopherol acetate per ewe per day for 30 days prior to lambing failed to prevent muscular dystrophy in lambs.[17] This report is a confirmation of a previous report that muscular dystrophy in lambs was not prevented by supplementation of the ration of the prepartum ewe with tocopherols.[41] A number of investigators have determined the tocopherol levels in plasma and in milk.[16, 24–29, 29–31, 34, 36–38, 40, 41]

One group of investigators demonstrated that the low level of total tocopherols in the milk of the ewes fed a ration of alfalfa and beans was the cause of the muscular dystrophy observed among the lambs.[41]

Preliminary investigation at this laboratory indicated a wide range of plasma and milk-tocopherol levels among ewes on various rations in a flock in which muscular dystrophy was occurring in the lambs. Therefore, this study was made in an attempt to correlate the tocopherol levels in the plasma and early milk of ewes with the rations fed and with the natural occurrence of muscular dystrophy in the lambs.

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EXPERIMENTAL PROCEDURE

A flock of 200 ewes on a farm in the Gallatin Valley, Montana, had been fed a home grown red clover hay and ½ lb. of cracked barley per head per day during the winter and spring of 1952–1953. The mortality in the lambs, born to these ewes in 1953 from "white muscle disease" was approximately 70 per cent. The remainder of the hay which had been fed to these ewes was purchased and was used as red clover hay in this experiment.

The alfalfa hay used was grown on another ranch in the "white muscle area" of the same valley. The alfalfa hay fed in the 1954 experiment was grown in 1953; that fed in the 1955 experiment was grown on the same ranch in 1954.

The pelleted protein supplement fed to the ewes in pen 9 was prepared for us by the Montana Flour Mills, Great Falls, Mont. It consisted of molasses, wheat bran, wheat middlings, low-grade

TABLE 1—Feeding Schedule of Ewes in the Study of Muscular Dystrophy

Pen (No.)	No. of ewes	Gestation First 136 days	Gestation Last 14 days	Lactation First 18 days
1	11	← red clover hay →		
		← 0.5 lb. barley →		
2	11	← red clover hay →		
3	11	← 1953 alfalfa hay →		
		← 0.5 lb. barley →		
4	11	← 1953 alfalfa hay →		
			← 1.0 lb. oats →	
5	10	← 1953 alfalfa hay →		
			← 0.5 lb. barley →	
6	10	← 1953 alfalfa hay →	← red clover hay →	
7	10	← 1953 alfalfa hay →	← red clover hay →	
			← 0.5 lb. barley →	
8	31	← 1954 alfalfa hay →		
			← 0.5 lb. oats →	
9	31	← 1954 alfalfa hay →		
			← 0.5 lb. protein suppl. →	
			← 0.5 lb. oats →	
10	31	← 1954 alfalfa hay →		
			← 0.5 lb. protein suppl. →	
			← 0.0025 lb. DPPD →	
			← 0.5 lb. oats →	
11	10	These ewes were selected at random from the experimental range band of 2,000 ewes of the Montana State College. Muscular dystrophy had never been observed in this band. The ewes had grazed on native grass through the winter, and when the weather became severe, a mixed grass hay was fed.		

flour, ground yellow corn, pulverized oats, alfalfa leaf meal, soybean meal, linseed meal, cottonseed meal, stabilized vitamins A and D from fish liver oil, calcium, phosphorus, manganese sulfate, trace minerals (iron, cobalt, copper, iodine, zinc, and boron), and salt. The guaranteed analyses were a minimum of 20.0 per cent protein and 3.5 per cent fat, and a maximum of 10.0 per cent fiber and 10.0 per cent ash. The ewes in pen 10 received this same pelleted protein supplement, to which had been added 10 lb. of N,N'-diphenyl-p-phenylenediamine (DPPD) per ton. This compound, which is an antioxidant, was being tested by us as a possible preventive of muscular dystrophy at the request of Dr. William H. Burkitt of the Montana Flour Mills Co.

The ewes in this experiment were selected at random from the flock maintained by the Montana Veterinary Research Laboratory. They were grade ewes of Targhee, Columbia, and Rambouillet breeding. The ages of the ewes varied from 2 to 8 years with no 3-year-olds included.

For the gestation period of 1953–1954, 74 ewes were separated into seven pens (pens 1 to 7), and for the period of 1954–1955, 93 ewes were separated into three pens (pens 8 to 10). In addition, 10 range ewes (pen 11) were studied. The feeding of all these ewes is described (table 1).

At monthly intervals, starting three months prepartum, 40 ml. of blood was obtained from each ewe in pens 1 to 4 and 8 to 10. The blood was drawn into a vial containing 1 ml. of a saturated solution of sodium citrate. Plasma-tocopherol estimations were made on each sample using methods described by Quaife and Harris.[26] Analyses were corrected for the dilution of the blood by the citrate solution.

Milk samples were obtained from each ewe on postpartum days 1, 4, 11, and 18. Milk-tocopherol determinations were made on each sample according to methods described by Quaife,[26] and Quaife and Biehler.[27]

Clinical observations were made twice daily for

TABLE 2—Tocopherol Levels of Plasma and Milk of Ewes

	Plasma tocopherol (μg./100 ml.) Month prepartum				Milk tocopherol (μg./100 ml.) Day postpartum			
	3	2	1	0	0	4	11	18
PEN 1 (53% OF LAMBS AFFECTED) 1953–1954								
Fed red clover hay and barley from breeding time through postpartum day 18								
All 11 ewes with 15 lambs	268 (135–435)	297 (97–466)	359 (153–846)	160 (67–458)	530 (139–944)	337 (42–735)	247 (97–611)	151 (56–458)
Eight ewes with 8 dystrophic lambs	242 (135–426)	331 (214–466)	382 (216–846)	121 (67–189)	502 (139–749)	195 (42–735)	252 (97–611)	172 (69–458)
Three ewes with 7 normal lambs	335 (282–435)	203 (97–380)	296 (153–402)	264 (92–458)	601 (334–944)	517 (463–549)	226 (97–356)	76 (56–97)
PEN 2 (56% OF LAMBS AFFECTED) 1953–1954								
Fed red clover hay from breeding time through postpartum day 18								
All 11 ewes with 15 lambs	216 (120–417)	237 (72–500)	330 (139–944)	141 (53–205)	725 (305–1330)	264 (111–555)	258 (139–694)	375 (83–1041)
Eight ewes with 9 dystrophic lambs	231 (126–417)	266 (86–500)	388 (167–944)	146 (122–205)	771 (305–1330)	241 (139–463)	243 (139–694)	111 (83–139)
Three ewes with 6 normal lambs	176 (120–225)	118 (72–164)	176 (139–250)	127 (53–164)	541 (416–666)	333 (111–555)	361	638 (236–1041)
PEN 3 (45% OF LAMBS AFFECTED) 1953–1954								
Fed alfalfa hay and barley from breeding time through postpartum day 18								
All 11 ewes with 20 lambs	267 (177–513)	270 (103–416)	381 (111–1082)	227 (150–302)	859 (69–2081)	280 (111–708)	307 (42–1679)	257 (56–735)
Six ewes with 9 dystrophic lambs	238 (177–327)	261 (180–389)	291 (111–860)	221 (150–289)	532 (69–902)	397 (111–708)	471 (56–1679)	217 (56–569)
Five ewes with 11 normal lambs	301 (191–513)	279 (103–416)	488 (194–1082)	233 (178–302)	1252 (406–2081)	138 (111–194)	108 (42–167)	304 (97–735)
PEN 4 (0% OF LAMBS AFFECTED) 1953–1954								
Fed alfalfa hay from breeding time and oats 2 weeks prepartum through postpartum day 18								
All 11 ewes with 14 lambs	.....	.....	530 (111–1415)	242 (111–513)	477 (111–888)	347 (56–708)	140 (69–250)	155 (42–319)
PEN 5 (13% OF LAMBS AFFECTED) 1953–54								
Fed alfalfa hay and barley from parturition time through postpartum day 18								
All 10 ewes with 15 lambs	.....	.....	.....	.....	960 (208–2398)	328 (42–916)	222 (42–694)	155 (42–278)
Two ewes with 2 dystrophic lambs	.....	.....	.....	.....	633 (600–666)	271 (42–500)	139 (42–236)	180 (153–208)
Eight ewes with 13 normal lambs	.....	.....	.....	.....	1042 (208–2398)	342 (111–916)	243 (56–694)	148 (42–278)
PEN 6 (31% OF LAMBS AFFECTED) 1953–1954								
Fed red clover hay from parturition time through postpartum day 18								
All 10 ewes with 16 lambs	.....	.....	.....	.....	768 (361–2054)	483 (83–1804)	137 (42–250)	156 (97–291)
Five ewes with 5 dystrophic lambs	.....	.....	.....	.....	732 (361–1249)	766 (125–1804)	157 (83–250)	156 (111–278)
Five ewes with 11 normal lambs	.....	.....	.....	.....	802 (361–2054)	200 (83–365)	117 (42–185)	156 (97–291)

signs of muscular dystrophy. The diagnosis was confirmed at necropsy by gross and microscopic examination of affected tissue of those lambs in which muscular dystrophy was fatal.

RESULTS AND DISCUSSION

The results of the determinations of the tocopherol levels in the blood plasma and in the milk made on each ewe are given (table 2).

The 177 ewes in the 11 pens had 263 lambs, 64 of which developed muscular dystrophy. A summary of the distribution of the affected lambs is shown (table 3). Seventeen lambs died from muscular dystrophy. The diagnosis was confirmed at necropsy.

The average age at which signs of muscular dystrophy appeared in the lambs was 8.7 days, varying from five to 22 days. If the amount of tocopherol in the milk was a factor, the milk consumed prior to the ninth day should determine whether or not the lamb would develop muscular dystrophy. Therefore, in computing the average milk-tocopherol levels (graph 1), the values for day zero and postpartum day four were used. Although the incidence of muscular dystrophy in the lambs of the various pens varied from 56 per cent to none, there was no correlation of this incidence with the milk-tocopherol levels.

The tocopherol levels of milk, collected on the day of parturition from the 6 ewes in pen 3 with affected lambs, averaged 532 μg./100 ml.; the 5 ewes in the same pen with nonaffected lambs had a higher average of 1,252 μg./100 ml. This difference in the tocopherol level in the milk at parturition was significant (P.L., 0.01). However, contrary results were observed in the milk taken postpartum day four from ewes in pens 3 and 9. The average milk-tocopherol level in four postpartum samples from the 6 ewes with affected lambs in pen 3 was 397 μg./100 ml.; those from the 5 ewes in pen 3 with nonaffected lambs was low at 138 μg./100 ml. This difference in milk-tocopherol levels was significant (P.L., 0.05). The average tocopherol lev-

TABLE 2 (Continued)—Tocopherol Levels of Plasma and Milk of Ewes

	Plasma tocopherol (μg./100 ml.) Month prepartum				Milk tocopherol (μg./100 ml.) Day postpartum			
	3	2	1	0	0	4	11	18
PEN 7 (25% OF LAMBS AFFECTED) 1953–1954								
Fed red clover hay and barley from parturition time through postpartum day 18								
All 10 ewes with 15 lambs	.....	.....	.....	.....	813 (208–1915)	297 (83–1221)	161 (42–735)	174 (97–430)
Four ewes with 4 dystrophic lambs	.....	.....	.....	.....	850 (208–1721)	246 (83–652)	97 (42–125)	236 (97–430)
Six ewes with 12 normal lambs	.....	.....	.....	.....	787 (300–1915)	334 (83–1221)	204 (44–735)	131 (111–180)
PEN 8 (44% OF LAMBS AFFECTED) 1954–1955								
Fed alfalfa hay from breeding time and oats 2 weeks prepartum through postpartum day 18								
All 27 ewes with 48 lambs	.....	101 (14–669)	53 (0–153)	40 (0–83)	670 (28–2013)	216 (0–735)	194 (35–490)	160 (42–416)
Fifteen ewes with 21 dystrophic lambs	.....	112 (14–669)	49 (0–111)	37 (14–69)	632 (157–1430)	239 (0–735)	209 (40–405)	148 (67–416)
Twelve ewes with 27 normal lambs	.....	84 (14–167)	58 (14–153)	43 (0–83)	724 (28–2013)	183 (0–700)	174 (35–490)	178 (42–350)
PEN 9 (6% OF LAMBS AFFECTED) 1954–1955								
Fed alfalfa hay from breeding time and protein pellets and oats 2 weeks prepartum through postpartum day 18								
All 28 ewes with 47 lambs	.....	140 (14–569)	67 (0–291)	37 (7–132)	858 (120–5966)	186 (28–870)	192 (26–786)	193 (0–694)
Three ewes with 3 dystrophic lambs	.....	185 (14–444)	185 (14–291)	23 (7–42)	1046 (666–1471)	408 (125–870)	129 (26–296)	237 (167–278)
Twenty-five ewes with 44 normal lambs	.....	135 (14–569)	52 (0–278)	38 (7–132)	833 (120–5966)	156 (28–461)	200 (49–786)	186 (0–694)
PEN 10 (7% OF LAMBS AFFECTED) 1954–1955								
Fed alfalfa hay from breeding time and pellet with DPPD and oats 2 weeks prepartum through postpartum day 18								
All 29 ewes with 42 lambs	.....	114 (14–708)	74 (0–236)	35 (0–83)	533 (83–2479)	190 (35–833)	192 (0–923)	185 (38–444)
Three ewes with 3 dystrophic lambs	.....	93 (56–166)	42 (28–56)	40 (9–76)	337 (83–694)	150 (94–194)	110 (48–216)	159 (67–211)
Twenty-six ewes with 39 normal lambs	.....	116 (14–708)	76 (0–236)	34 (0–83)	556 (83–2479)	194 (35–833)	201 (0–923)	188 (38–444)
PEN 11 (0% OF LAMBS AFFECTED) 1953–1954								
Fed mixed native grass hay from breeding through postpartum day 18								
All 10 ewes with 15 lambs	.....	.....	.....	.....	945 (345–1650)	567 (45–1755)	102 (30–180)	108 (15–225)

DPPD—N,N'-diphenyl-p-phenylenediamine.

TABLE 3—Morbidity and Mortality of Lambs with Muscular Dystrophy

Year	Pen No.	No. of ewes	No. of lambs	Morbidity (%)	Mortality (%)
1954	1	11	15	53	13
	2	11	15	56	40
	3	11	20	45	10
	4	11	14	0	0
	5	10	15	13	6
	6	10	16	31	6
	7	10	16	25	6
1955	8	31	48	44	4
	9	31	47	6	2
	10	31	42	7	2
1953	11	10	15	0	0
Total		177	263		

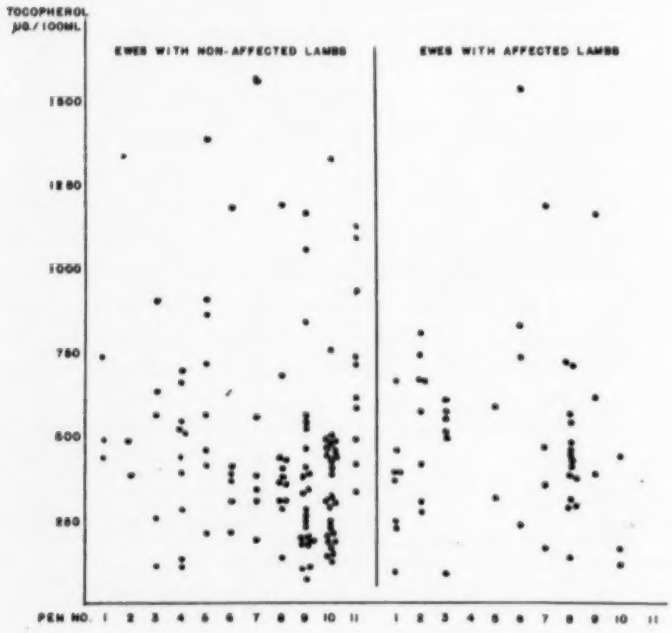
els from the milk from the ewes in pen 9, taken on the fourth postpartum day, were 408 μg./100 ml. for the 3 ewes with affected lambs and only 156 μg./100 ml. for the 25 ewes with nonaffected lambs. The difference in milk-tocopherol levels was significant (P.L., 0.05). Other than those three contradictory differences in average milk-tocopherol levels, there were no significant differences in the average tocopherol levels between the ewes with affected lambs and those with nonaffected lambs. Other seemingly great differences between average tocopherol levels of ewes with affected lambs and ewes with normal lambs (for example, on postpartum day 4 in pens 1 and 6) are not statistically significant because of the very wide range of values used in calculating these averages.

There was no significant difference in the tocopherol levels of the milk of the ewes fed red clover hay, alfalfa, or mixed grass hay. Supplementation with barley, oats, protein pellets, or DPPD, as supplied in this experiment, had no significant effect on the milk-tocopherol levels.

It is concluded, in this study, that the level of milk-tocopherol of ewes was not related to the incidence of muscular dystrophy in their lambs.

There is no correlation of the morbidity of the lambs and the tocopherol levels in the plasma of their dams at 1 and 0 months prepartum either by pens or by individuals (graph 2). There are no correlations of plasma-tocopherol levels and the rations fed in 1954 or of the plasma-tocopherol levels and the rations fed in 1955. The tocopherol levels in the plasma were lower in the three pens in 1955 than in the four pens fed in 1954; the levels decreased in all pens as pregnancy advanced.

The supplementation of barley to the alfalfa hay ration of the ewes appears to be a factor in producing muscular dys-


TOCOPHEROL μG./100ML
EWES WITH NON-AFFECTED LAMBS
EWES WITH AFFECTED LAMBS
1500
1250
1000
750
500
250
PEN NO. 1 2 3 4 5 6 7 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11

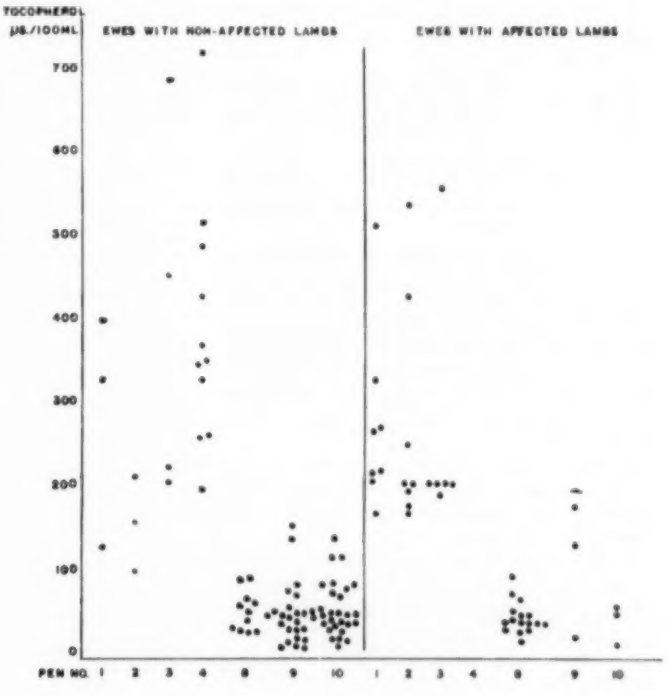
Graph 1—Tocopherol levels in the colostrum (average of first and fourth days) of ewes whose lambs did and did not develop muscular dystrophy.

trophy in the lambs. There was no muscular dystrophy in the lambs in pen 4 where the ewes were fed alfalfa hay and oats. However, in pens 3 and 5, where the ewes received the same alfalfa hay, plus barley, lamb morbidity was 45 per cent in pen 3, where barley was supplemented from breeding time, and 13 per cent in pen 5 where barley was fed from parturition time. The lambs in pen 4 did receive oats, which was not fed to those in pens 3 and 5. Those in pens 8, 9, and 10 all received oats from parturition time, with no apparent effect on the rate of morbidity of muscular dystrophy. Supplementation of barley to red clover hay had no apparent effect on the morbidity.

There was a correlation between the length of time the "white muscle disease rations" were fed to the ewes and the morbidity rate in the lambs with muscular dystrophy. The red clover hay and barley fed the ewes in pen 1, the red clover hay fed the ewes in pen 2, and the alfalfa and barley fed the ewes in pen 3, starting at breeding time, resulted in morbidity of 53, 56, and 45 per cent, respectively; whereas, the same rations fed ewes in pens 5, 6, and 7, starting at parturition time, produced morbidity of 13, 31, and 25 per cent, respectively.

The alfalfa hay fed the ewes in pen 8 resulted in a lamb muscular dystrophy morbidity of 44 per cent. A pelletted protein supplement was added to the alfalfa ration fed to the ewes in pen 9 where the morbidity was 6 per cent. The ewes in pen 10, receiving alfalfa hay supplemented with a protein pellet containing 10 lb. of DPPD per ton, had a morbidity of 7 per cent. This indicates that DPPD was not a factor in reducing the incidence of muscular dystrophy because the same protein pellet without the DPPD reduced the morbidity as much. Some other factor or factors in the protein pellet apparently was responsible for the lower incidence of muscular dystrophy.

The effect of age of the ewes on the incidence of muscular dystrophy in their lambs is shown by the following percentages: 8 year-olds, 35; 7 year-olds, 18; 6 year-olds, 35; 5 year-olds, 30; 4 year-olds,



Graph 2—Tocopherol levels in the blood plasma (average of values determined in 1 and 0 months prepartum) of ewes whose lambs did and did not develop muscular dystrophy.

12; and 2 year-olds, 25; thus, there was no correlation between the occurrence of muscular dystrophy in lambs and ages of ewes.

SUMMARY AND CONCLUSIONS

1) Determinations of the tocopherol levels in the plasma were made on 406 blood samples from 128 pregnant ewes fed hay from a "muscular dystrophy area." In 1954, these values averaged 250, 244, 364, and 193 μg./100 ml. for 3, 2, 1, and 0 months prepartum, respectively. In 1955, the averages were 118, 65, and 37 μg./100 ml. for 2, 1, and 0 months prepartum.

2) Determinations of the tocopherol levels were made on four milk samples collected on postpartum days 1, 4, 11, and 18 from 177 ewes fed hay from a "muscular dystrophy area." The average levels at these four stages of lactation were 732, 335, 210, and 189 μg./100 ml. in 1954. In 1955, they were 683, 197, 193, and 179 μg./100 ml. The corresponding values from 10 range ewes kept on a "nondystrophy area" in 1953 were 945, 567, 102, and 108 μg./100 ml. There were occasional, apparently random, values which differed markedly from these averages.

3) The morbidity of the lambs with muscular dystrophy in 11 groups varied from 0 to 56 per cent.

4) The average age of the first signs of muscular dystrophy in the lambs was 8.7 days, varying from 3 to 22 days.

5) There was no correlation of the tocopherol levels in the milk and in the plasma of the ewes with the incidence of muscular dystrophy in lambs.

6) There was no significant difference in tocopherol levels in the milk and in the plasma of the ewes fed red clover, alfalfa, and mixed grass hays. The addition of barley, oats, protein supplement, and N,N′-diphenyl-p-phenylenediamine to the hay rations resulted in no significant difference in the plasma and milk-tocopherol levels of the ewes.

7) The length of time a "white muscle disease ration" was fed ewes apparently increased the incidence of muscular dystrophy in the lambs.

8) The addition of a protein supplement to the ewe's ration decreased the morbidity of the lamb with muscular dystrophy from 44 to 6 or 7 per cent.

9) There was no significant correlation between the incidence of muscular dystrophy in lambs and the ages of ewes.

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Abortion and Pre- or Postnatal Death of Young Due to *Listeria Monocytogenes*. III. Studies in Ruminants

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IT WAS shown by Gray *et al.*[1,2] that abortion and pre- or postnatal death of young rabbits could be produced both by conjuctival instillation of *Listeria monocytogenes* and by addition of the bacterium to the drinking water of the pregnant doe. The lesions produced in the fetuses, and especially in the young born at term, appeared to be similar, if not identical, to those described for natural infection in both animal and human populations.[3] Similar results could be produced by addition of suspensions of *L. monocytogenes* to the drinking water of pregnant sheep and goats. Although the number of animals for study was small, the uniformity of results, especially in view of similar results in rabbits,[1,2] appears to justify publication of these preliminary findings.

MATERIALS AND METHODS

Two pregnant ewes, 3 pregnant goats, 1 castrated male goat, and 1 cow in the seventh month of gestation were exposed to *L. monocytogenes* by adding suspensions of the bacterium to the drinking water. The culture employed had been isolated originally from the medulla oblongata of a calf which died of listeric encephalitis complicated by vitamin E deficiency. Prescription bottles (32-oz. sani-glas), containing approximately 100 ml. of tryptose agar (Difco), were inoculated with a distilled water suspension of freshly grown culture and incubated at 37 C. for 18 to 24 hours. They were then filled with tap water and agitated to free the culture from the agar surface. Each day, 64 oz. of this suspension was added to approximately 10 qt. of tap water in a galvanized pail. This constituted the only source of fluid intake for the ewes and goats for seven days. For the cow, 128 oz. of a similar suspension were added daily to approximately nine gal. of tap water for 14 days.

Three goats, 1 near termination of pregnancy, 1 nonpregnant, and 1 castrated male were exposed by conjunctival instillation to the same culture of *L. monocytogenes*. It was grown on tryptose agar (Difco) slants at 37 C. for 18 to 24 hours. It was then suspended in sterile distilled water to a density approximating the number 10 tube of the McFarland nephelometer when compared in a Cenco-Sheard-Sanford photelometer. Four drops of this suspension were instilled into the conjunctival sac of the left eye by means of a capillary pipette. Later, necropsies were done on all aborted fetuses, on all young which died or were destroyed, and on all dams which died or were destroyed, and the liver, spleen, kidney, lungs, heart blood, stomach contents, urine, brain and spinal fluid were cultured as described by Gray *et al.*[4,5] Tissues to be cultured were macerated in a mortar together with approximately 10 ml. of sterile distilled water. A large loopful (4.0 mm. dia.) of this suspension was plated on tryptose agar (Difco) plates. The remaining suspension was stored in the refrigerator at 4 C. The plates were incubated at 37 C. for 18 to 24 hours after which they were examined by means of a binocular scanning microscope and obliquely transmitted light. Tissue suspensions which failed to reveal the presence of *L. monocytogenes* were replated at intervals of several weeks. If this organism was not detected after three months of refrigeration, the suspension was considered negative.

RESULTS

Oral Exposure.—Ewes.— Six days after exposure to *L. monocytogenes*, 1 ewe gave birth to what appeared to be slightly premature, stillborn twin lambs. At necropsy, extensive hemorrhages were revealed in the kidneys and lymph nodes of both lambs. There were hemorrhages in the liver and intestine and congestion of the brain in 1 lamb, and a few petechial hemorrhages in the lungs and epicardium, and mottling and congestion of the liver in the other. *Listeria monocytogenes* was isolated from the viscera (table 1). During the next nine days, the ewe showed a thick blood-tinged, mucus-like, putrid discharge from the vagina (fig. 1). She was anoretic, became very weak, emaciated, and dyspneic, and expired on the ninth postpartum day. There were no symptoms suggestive of damage to the central nervous system. At necropsy, the uterus was necrotic and filled

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TABLE 1—Number of Positive Cultures of Listeria Monocytogenes from Lambs and Kids

		Liver	Stomach contents	Kidney	Brain	Lungs	Blood	Spleen
Lambs								
Aborted	1	+	—	—	....	—	—	—
	2	+	+	—	+	—	+	—
Born alive		+	—	+	+	—	—	....
Kids								
Dam died	1	—	—	—	—	—	—	—
	2	+	+	—	+	—	....	....
	3	+	+	+	+	+	....	....
Aborted 12 days postexp.	1	+	+	+	+	+	+	....
	2	+	+	+	+	+	+	....
Aborted 13 days postexp.	1	+	+	+	+	+	+	....
	2	+	+	+	+	+	+	....
	3	+	+	+	+	+	+	....
Born to dam in contaminated pen								
Stillborn		—	—	+*	+*	—	—	—
Destroyed		—	—	—	—	—	—	—

* After 66 days of refrigeration.

with a thick, putrid, almost black, material and contained necrotic cotyledons (fig. 2). The liver and kidneys were yellowish and very friable. There were no other lesions. *Listeria monocytogenes* was isolated on primary culture only from the uterus and udder. However, it was isolated from the liver after 49 days of refrigeration and from the brain after 75 days.

Seven days after exposure, the second ewe gave birth to what appeared to be a full-term lamb. It was weak, stood with difficulty, and was never seen suckling the ewe. It became progressively weaker and displayed signs of central nervous system involvement. These included twitching of the facial muscles and forelegs, incoordination, and torticollis. It expired on the eighth postnatal day (fig. 1). Necropsy revealed only slight pneumonia of the apical lobes. *Listeria monocytogenes* was isolated from the viscera (table 1). Initial

Fig. 1—Ewe showing thick, blood-tinged, mucus-like, putrid discharge from the vagina nine days after giving birth to premature, stillborn twin lambs; and (below) the second ewe's lamb which expired when eight days old (day photograph was taken).

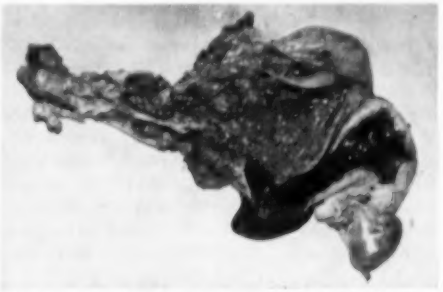

Fig. 2—The uterus of the ewe shown in figure 1 was necrotic and filled with a thick, putrid, almost black, material and contained necrotic cotyledons.

cultures from the cotyledons failed to reveal its presence but the cultures became positive after refrigeration for 26 days. The ewe showed no signs of illness at any time following exposure. Swab cultures taken from the vagina failed to reveal the organism. The ewe was destroyed one month after the lamb was born and necropsy revealed an incompletely involuted uterus which was filled with what appeared to be caseated pus. There were no other lesions and all cultures remained sterile (table 2).

Goats.—Of the 3 pregnant goats exposed orally to *L. monocytogenes*, 1 was listless on the eighth postexposure day and

TABLE 2—Summary of Results

Group	No. of anim.	Fate of animals
Orally exposed		
Ewes		
Exposed near term	2	1 premature expulsion of stillborn twins; died
		1 weak, full-term lamb; died; ewe survived but destroyed
Goats		
Exposed in approx. fourth gestation month	3	1 died
		2 aborted and survived; 1 destroyed
Exposed by contact near end of fourth gestation month	1	Full-term triplets, 1 stillborn, 2 survived; dam survived
Conjunctivally exposed		
Exposed 27 days prepartum	1	Full-term triplets by cesarean section; dam died with listeric encephalitis two days postpartum

died two days later. Necropsy revealed a greatly distended uterus which contained 3 fetuses at about the fourth month of gestation. The cotyledons were necrotic. Pulmonary edema, yellowish discoloration of the liver, and a small amount of mucopurulent exudate in the vagina were also present. All other viscera appeared normal. The organism was isolated on primary culture only from the liver and certain cotyledons. Some cotyledons contained the organism in large numbers while cultures prepared from others remained sterile. Cultures from the kidney and the brain became positive for *L. monocytogenes* after 36 days of refrigeration. Necropsy of the fetuses revealed only pale yellowish livers. The organism was isolated from several organs (table 1).

Fig. 3—A fetus, about the fourth month of gestation, aborted from a goat which had been exposed orally to Listeria monocytogenes.

The other 2 goats showed no indications of illness following oral exposure to *L. monocytogenes*. However, 1 aborted twins, and the other aborted triplets on the twelfth and thirteenth postexposure days, respectively. These 5 fetuses appeared to be in about the fourth month of gestation (fig. 3). Only 1 appeared to have been dead for a time before expulsion. A few small yellow necrotic foci were observed in the livers of the other 4 (fig. 4). There were no other detectable lesions. Isolations of *L. monocytogenes* are shown in table 1.

The pen in which the 3 goats were kept was not cleaned following the two abortions. However, the watering trough was thoroughly cleaned and disinfected. Culture of the fetal fluids suggested that this relatively small area (7 ft. by 8 ft. 2 in.) might be highly contaminated. Five days after the last abortion a nonexposed goat, late in gestation, was put in the pen with the 2 surviving goats which had aborted. Twenty-one days after being placed in the contaminated pen, she gave birth to triplets, 1 of which was stillborn. Necropsy of this kid revealed no detectable pathological alterations. Primary cultures from the fetal membranes and fluids and from the kid failed to show the presence of *L. monocytogenes*. Nevertheless, the organism was isolated from the brain and the kidney after 66 days of refrigeration (table 1). These results are summarized in table 2.

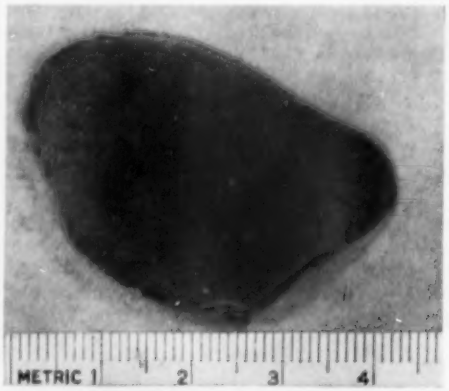

Fig. 4—A liver from an aborted fetus of a goat, exposed orally to Listeria monocytogenes, showing a few small yellow necrotic foci.

The goat which aborted 13 days after exposure was destroyed seven days later. Lesions were confined to the uterus. The

endometrium was congested and covered with pus. *Listeria monocytogenes* was isolated only from this pus. The 2 surviving goats showed no postpartum ill effects to exposure to the organism. Both are presently pregnant.

The castrated male goat showed no ill effects following the addition, for seven days, of *L. monocytogenes* to the drinking water. However, when the animal was destroyed three days later, necropsy revealed a hemorrhagic enteritis and focal necrosis of both kidneys. The organism was isolated on primary culture from the urine, kidney, and left nostril.

Cow. — The cow, orally exposed to *L. monocytogenes* for two weeks during the seventh month of gestation, showed no evidence of illness. She delivered a normal full-term calf 45 days after the culture was removed from the drinking water. Cultures of the fetal fluids and membranes failed to reveal the organism.

Conjunctival Instillation.—Goats.—Two days following the instillation of *L. monocytogenes* into the conjunctival sac of a female goat, late in gestation, the eye developed a slight serous discharge which persisted for two days, after which the eye appeared normal. At no time did the goat appear ill but 26 days following exposure she appeared to be in labor. The following day she was prostrate, unable to get up, and a cesarean section was performed under pentothal anesthesia. Three apparently healthy full-term kids were delivered.* The dam was given 12 million units of penicillin and appeared to be resting quietly. Three cultures from the cotyledons and amniotic fluid remained sterile. The kids suckled colostrum in the evening and again late at night. The following day the kids appeared normal and suckled a little milk from the dam. However, the dam appeared quite ill and by midmorning showed signs of central nervous system involvement, incoordination, convulsions, twitchings of the facial muscles, and running motions, suggestive of listeric encephalitis (fig. 5). Body temperature was 106.5 F. In the evening, she was given 1,000 ml. of cow's milk but no medication. The next day her temperature was 104,

Fig. 5—A female goat showing signs of listeric encephalitis after conjunctival instillation of Listeria monocytogenes.

she was quiet and calm, and she drank 500 ml. of cow's milk. However, she made no attempt to get up, and died approximately 56 hours after the operation. At necropsy, the uterus contained a small amount of caseous purulent exudate and a few necrotic cotyledons. Some cotyledons appeared normal. There were no other lesions. *Listeria monocytogenes* was isolated on initial culture from the brain and liver. One of the 3 kids was destroyed for necropsy on the seventh postpartum day. Cultures revealed nothing of significance. Another kid, destroyed at the age of 5 months due to an injury, showed no lesions and all cultures remained sterile. The remaining kid is presently 8 months old and apparently normal.

One nonpregnant female and a castrated male showed slightly increased lacrimation in the exposed eye for three to four days following instillation. After this, the eye appeared normal and there was no indication of illness in either animal. The castrated male was destroyed for necropsy on the twenty-fifth postexposure day and cultures revealed nothing of significance.

Discussion

The results revealed that the reproductive system of pregnant sheep and goats was highly susceptible to infection by orally introduced cultures of *L. monocytogenes*. If exposure was early in gestation, the young were aborted and the organism was readily isolated from most of the viscera. If exposure was near term, the young were either stillborn or survived only a few days. The young were always fatally infected, even though in some instances the dam displayed no signs of illness. It appeared that if the infected conceptus was expelled quickly and completely the

* The authors thank Dr. D. H. McWade, Department of Animal Pathology, Michigan Agricultural Experiment Station, for performing the operation.

dam suffered only a localized metritis. However, if the infected conceptus was retained, a generalized infection resulted and the dam died with listeric septicemia. These results were almost identical to those reported by Gray *et al.*[2] for rabbits. Of 57 full-term rabbits born to 8 does orally exposed from five days to 30 hours prepartum, 32 were stillborn and none survived more than five days. Similar results in rabbits and a goat were reported by Hahnefeld[6] and Hahnefeld and Nisolk.[7] Their findings and those of the present study strongly suggest that the gravid uterus and, especially, its contents are highly susceptible to infection with *L. monocytogenes*. A male goat appeared relatively refractory to this method of exposure but was found to harbor *L. monocytogenes* in the kidneys and urine.

The entire uterine contents may not be equally infected. This was observed in several instances but was most strikingly demonstrated by the fetuses of the goat which died following oral exposure. *Listeria monocytogenes* was isolated (table 1) from all viscera of 1 fetus; from only the liver, stomach contents, and brain of 1; while all cultures from the third fetus failed to show significant bacterial growth. Likewise, of several cotyledons from the same animal, some contained *L. monocytogenes* in large numbers while cultures of others remained sterile. This was also true but to a lesser extent, in the studies on rabbits.[1,2] It has not been determined satisfactorily whether the organism actually was not present or whether this is a reflection of inadequacies in isolation technique.

The results also emphasize the need for maceration and refrigeration of tissues when initial cultures of suspect material fail to reveal the presence of *L. monocytogenes*. Of 12 isolations of the organism from adult animals, seven were positive on initial culture and five were positive only after periods of refrigeration up to 75 days. This strongly suggests that *L. monocytogenes* often may be missed if only the usual isolation techniques are employed. This was demonstrated by the stillborn kid born to the nonexposed goat placed in the infected pen. Without refrigeration of the tissue suspensions, the presence of *L. monocytogenes* in the brain and kidney would never have been detected. From this, it appears that the organism may play a more important role in the pre- or postnatal death of young than has been suspected to date.

The stillbirth from the nonexposed goat demonstrated that inapparent infection could be established by direct contact with a contaminated environment. This is one of the few recorded instances of the direct experimental transmission of any form of listeriosis by contact with either known infected animals or environment. Although only 1 animal was involved, it does suggest that infection can be transmitted by contact with infected material. Vendrov[8] reported that listeriosis recurred each year in certain private piggeries in Siberia. If healthy pigs were placed in the contaminated pens they became infected, but it was not indicated whether listeriosis in pigs exposed in this manner was confirmed by isolation of *L. monocytogenes*. It was stated that infection was transmitted through excrement but not by contact with sick or healthy appearing animals. "Large ruminants" were not visibly affected by a similar exposure. Dedie[9] found increased antibody titers in noninfected chickens in contact with artificially infected birds. However, all birds exposed by contact remained asymptomatic, and the actual presence of *L. monocytogenes* could not be demonstrated.

Listeric abortion is perhaps the most hazardous form of listeric infection from the standpoint of both animal and human health. Van der Schaaf[10] reported a fatal listeric meningitis in a farmer following contact with an aborted bovine conceptus and a low-grade listeric septicemia in a veterinarian following a similar contact. The results of the present study revealed that not only was the aborted material highly contaminated, but also that *L. monocytogenes* was often found in the urine of the infected dam. Unfortunately, the feces were not cultured, but it is strongly suspected that this was also contaminated, particularly since the animals were exposed orally. Gray *et al.*[4] showed that *L. monocytogenes* readily passes through the intestinal tract of orally exposed rabbits. Vendrov[8] claimed that fresh manure from naturally infected pigs was highly infective. Although experimental evidence is presently lacking, it is probable that it may also pass through the intestinal tract

of ruminants. Animals which suffer listeric abortion should be regarded as highly contaminated.

The postmortem isolation of *L. monocytogenes* from the milk of 1 animal suggests that this may constitute an additional source of infection for the newborn. Gray and Thorp [11] found that when young rabbits born to noninfected does were allowed to suckle does exposed orally either two to three days prepartum or immediately postpartum, the young often died a few days days later with listeric septicemia. These findings establish the possible transmission of *L. monocytogenes* by milk from an infected animal. Wramby [12] and Potel [13] each report the isolation of *L. monocytogenes* from cow's milk. This also constitutes an additional health hazard since several investigators [14-17] claim that *L. monocytogenes* may survive pasteurization. Whether some of the sporadic outbreaks of listeric infection in man might be traced to contaminated milk remains to be determined but was suggested early by Burn.[18] Recently Potel [13] reported direct infection of a pregnant woman by drinking raw milk from a cow with an "atypical mastitis." *Listeria monocytogenes* was isolated in one instance from the milk of this cow. The woman gave birth to stillborn twins prematurely, and *L. monocytogenes*, of the same serotype as isolated from the milk, was isolated from the liver of each. From this it appears that milk as a source of listeric infection is in need of further investigation.

The results with the pregnant cow which was exposed orally to large doses of *L. monocytogenes* for two weeks, which failed to abort or show signs of illness, are difficult to explain, particularly in view of the results with sheep and goats. Exposure was made in the period of gestation in which listeric abortion most commonly occurs in cattle.[8] The infective dose was presumably much higher than an animal would experience under natural conditions. However, it is possible that hormonal levels may also play a part in listeric abortion. From published reports, it appears that listeric abortion in cattle is more common than in other species.[8] However, this may only reflect the greater emphasis placed on bovine abortions of all types. Both Paterson [19] and Hahnefeld [20] failed to produce abortion in pregnant sheep following oral exposure to *L. monocytogenes*.

The goat which died of listeric encephalitis 30 days following conjunctival exposure was of interest. Of 20 pregnant rabbits exposed in this manner by Gray *et al.*,[1] none displayed signs of encephalitis such as reported by Asahi and Hosoda,[21] both in presumably nonpregnant rabbits and goats following conjunctival instillation of *L. monocytogenes*. Usually, such instillation in the nonpregnant animal results only in what appears to be a localized conjunctivitis but exceptions have been reported by Graham *et al.*[22] and Gray *et al.*[4] who reported listeric encephalitis in a pig and a rabbit, respectively, following conjunctival instillation. Until further studies can be carried out on pregnant ruminants, the significance of this one instance can not be determined.

The results of this study establish the oral route as a possible mode of entry for *L. monocytogenes* and reveal that exposure by this method may cause intrauterine infection in pregnant sheep and goats. It does not imply that this is the only portal of entry leading to intrauterine infection, particularly since a similar infection followed conjunctival instillation in rabbits.[1] A venereal mode of infection is suggested by several reports. Stenberg [23] isolated *L. monocytogenes* from the vaginal mucus of a cow. Gray and McWade [24] isolated the bacterium from the cervix of a so-called repeat-breeder cow. Wenkebach [25] isolated *L. monocytogenes* from the urethral exudate of 5 men with gonorrhea and Stoot [26] reported its isolation from the cervix of a woman. Although the microorganism appeared to have no pathogenic role in any of these instances, the observations suggest the possibility of venereal transmission. Osebold and Inouye [27] reported fetal death and embryo resorption in rabbits exposed intravaginally on the seventh day of gestation. None of the does died or showed marked indications of illness. Unpublished material from this laboratory has revealed that rabbits exposed intravaginally during the third week of gestation may abort. This was not a consistent finding but emphasizes the possibility of venereal infection.

The misconception that listeriosis is principally a disease of the central nervous system has done much to impede the unravelling of the pathogenesis of the sev-

eral forms of the disease. The findings of Pallaske[28] and Shimizu *et al.*[29] suggest respiratory rather than oral exposure in listeric encephalitis of ruminants. In sheep and goats, it is most prevalent during the winter when the ewes are pregnant. If animals contract listeric encephalitis orally, it is difficult to explain, in view of the results of the present study, the relatively low incidence of abortion and pre- and postnatal death of young in a population consisting largely of pregnant animals.

SUMMARY

When suspensions of *Listeria monocytogenes* were added to the drinking water of 2 pregnant ewes and 3 pregnant goats, the young suffered a fatal listeric infection manifested by abortion if exposure was early in gestation, or by death of the young within the first postnatal week if exposure was near term. One ewe and 1 goat died of listeric septicemia following exposure. The surviving 3 animals and a male goat were not visibly affected by a similar exposure. The lesions in the aborted fetuses and the young born at term appeared to be identical to those seen in natural listeriosis. *Listeria monocytogenes* was isolated from most of the aborted fetuses and membranes. Of 57 isolations of *L. monocytogenes*, seven were positive only after the tissue suspensions were refrigerated for periods up to 75 days. One of triplets born to a nonexposed goat placed in a contaminated pen was found to harbor *L. monocytogenes* in the kidney and the brain. A cow in the seventh month of gestation was not affected by a similar exposure. One goat late in gestation exposed conjunctivally gave birth to apparently normal triplets but died of listeric encephalitis 56 hours postpartum. Listeric abortion appears to be one of the most hazardous forms of listeric infection from the public health viewpoint.

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Plasma, Urine, and Bile Levels of 17-Hydroxycorticosteroids in Sheep and Cattle Following the Administration of Hydrocortisone

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SEVERAL metabolic studies have demonstrated that the intravenous administration of hydrocortisone or cortisone to men, dogs, and rats produces high plasma titers of the steroid, followed by a rapid disappearance.

The investigations of Tomizawa *et al.*[1] and Nelson[2] on rats and dogs, respectively, have shown that the kidney is not involved in the rapid decline of plasma hormone in these species. According to these workers, the liver is the main factor in determining the plasma titer of exogenous hydrocortisone. The former group observed a slow decline in plasma hormone after rats had been "hepatectomized." Nelson, by simultaneously analyzing arterial and hepatic venous blood of dogs, found evidence for hydrocortisone uptake by the liver. Hyde and Williams[3] injected hydrocortisone-4-C^{14} intravenously into rats and found 84 per cent of the radiocarbon in the bile within four hours after injection. In contrast to this mode of excretion in the rat, Sandberg *et al.*[4] found that the human kidney was the major route for radiocarbon excretion after the administration of hydrocortisone-4-C^{14}. From these studies, it would appear that the main excretory route for both intact and metabolized hydrocortisone varies among species and oscillates between bile and urine depending on species peculiarities.

Wynne[5] studied some aspects of endogenous hydrocortisone excretion in sheep. He found no corticosteroids in a 24-hour collection of ewe feces and only traces of hydrocortisone in the urine of ewes injected with adrenocorticotrophic hormone (ACTH). Pooled bile from normal sheep contained 15 μg. of hydrocortisone per liter. Bile from an animal *in extremis* contained 600 μg. per liter.

In the course of investigations on adrenal steroid metabolism in ruminants, we have demonstrated that free and glucuronide-conjugated 17-hydroxycorticosteroids do not constitute a significant fraction of the urinary steroids in normal cows.[6] Observations in this laboratory substantiate those of Wynne[5] relative to the equally low levels of these steroids in sheep urine. Thus far, no study has been reported with respect to the metabolism of exogenous hydrocortisone in ruminants and, so far as the authors are aware, no report has been made on the combined biliary and urinary levels of the intact steroid in any animal after the administration of the steroid. This report deals with the plasma concentrations, rate of clearance, and excretory routes of intact hydrocortisone after the intravenous administration of the hormone to sheep and cattle.

METHODS

Animals used in this study were: 5 adult ewes, 1 ruminating wether lamb, 1 day-old ewe lamb, 2 ruminating heifer calves, 3 mature cows, 1 11-day-old bull calf, and 1 newborn bull calf.

The hydrocortisone for injection was prepared in an alcoholic solution and diluted to 25 cc. with normal saline for all animals except the newborn lamb to which a 10-cc. volume was administered. The dosage of the steroid was either 0.5 or 1.0 mg. per pound. In all trials, preinjection blood samples were taken for the establishment of baseline data. The hormone solution was then injected into the jugular vein for approximately one minute, and blood samples were taken at suitable intervals thereafter.

Eight-hour urine output was collected from 4 ewes, 2 heifer calves, 2 bull calves, and 1 cow. Collections were made at intervals in order to measure the time factor in urinary elimination. Urine was collected from bovine animals by use of the urination reflex and from ewes by means of an indwelling catheter.

For the experiments in bile collection, 1 ewe and 1 wether were fasted for three days prior to surgery. Under pentobarbital anesthesia, an incision was made in the flank just posterior to, and parallel with, the last right rib. The common bile duct was ligated with minimal damage to the vascular supply of the gall bladder. The hepatic duct was cannulated with a fiber dog catheter, the catheter being firmly anchored with nylon sutures. The cannula was exteriorized through a stab wound in the flank and was loosely sutured to the adjacent skin.

Upon recovery from anesthesia, the animals were fed *ad libitum* with good quality alfalfa hay. They were drenched daily with at least 500 cc. of Ringer's solution, and drinking water was replaced with Ringer's solution in order to make up the ionic deficit occasioned by the loss of bile. Recoveries were without incident. Bile flow was good initially and improved to a steady flow

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within 24 hours. Bile collections were made by attaching a plastic tube to the cannula. A hydrocortisone clearance trial was performed on the wether lamb on the second day after surgery. Ten days after the cannulization of this animal, the cannula became plugged. No attempt was made to clear the cannula even in the face of marked distress on the part of the animal. Although no outward signs of jaundice were observed over a period of three days, the animal became acutely ill and a second hydrocortisone clearance was run.

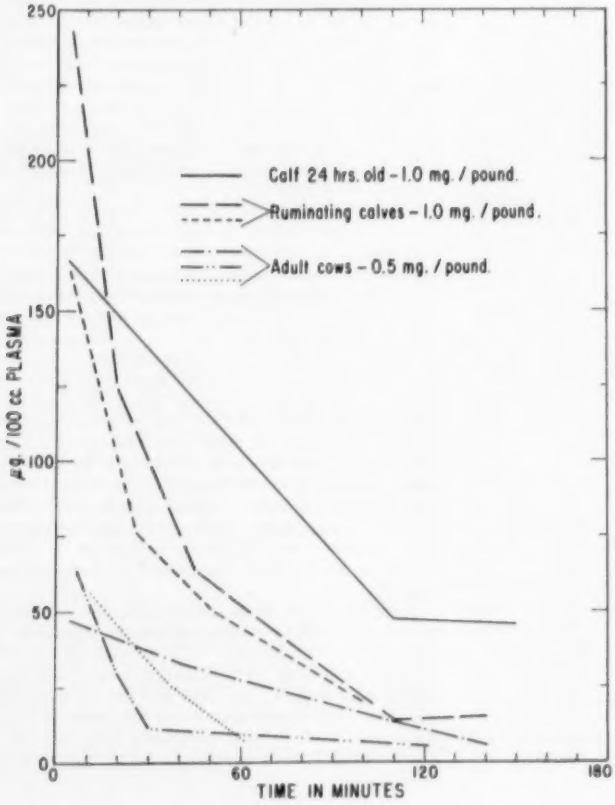
Plasma hydrocortisone was determined by the method of Nelson and Samuels.[7] This method proved to be more simple in execution than did those proposed by Bush and Ferguson[8] and Robertson and Mixner[9] for ovine and bovine plasma, respectively. Comparisons among the three methods gave identical recovery rates (93–109 %) when the free steroid was added to ovine or bovine plasma *in vitro*.

Hydrocortisone in the urine was determined by the method previously described.[6] The determination of the hormone and its conjugates in bile was accomplished by the same procedure.

RESULTS

The plasma clearance of injected hydrocortisone for all sheep and cattle is shown (graphs 1 and 2). Although the rate of disappearance of the free steroid from the plasma was related to the existing plasma titer as demonstrated by Eik-Nes *et al.*,[10] for man, the curves demonstrate that, at a given dosage, all normal ruminating sheep and cattle cleared their plasma to normal steroid values by two hours and 6 of 8 cleared by one hour. This is in contrast to results obtained on human subjects by Eik-Nes *et al.* After the intravenous administration of 50 mg. of hydrocortisone to these subjects, clearance of the steroid was not achieved for four to six hours.

Despite very high plasma levels immediately after the injection, newborn ruminants would seem to clear their plasma more slowly than do animals with a functional rumen. Although the newborn calf



Graph 1—Hydrocortisone levels of bovine plasma after intravenous injection of hydrocortisone.

and lamb received identical dosages of hydrocortisone when calculated on a weight basis, the lamb cleared its plasma more rapidly than did the calf.

The clearance of the wether that had undergone biliary cannulation 48 hours preceding the first trial was the slowest of all ruminating sheep. Impaired clearance of the hydrocortisone was very obvious when the second trial was run after the plugging of the cannula. The slow clearance in this case would seem to correlate with necropsy findings of a dilated hepatic duct, marked bile stasis, and early fibrotic changes in the liver.

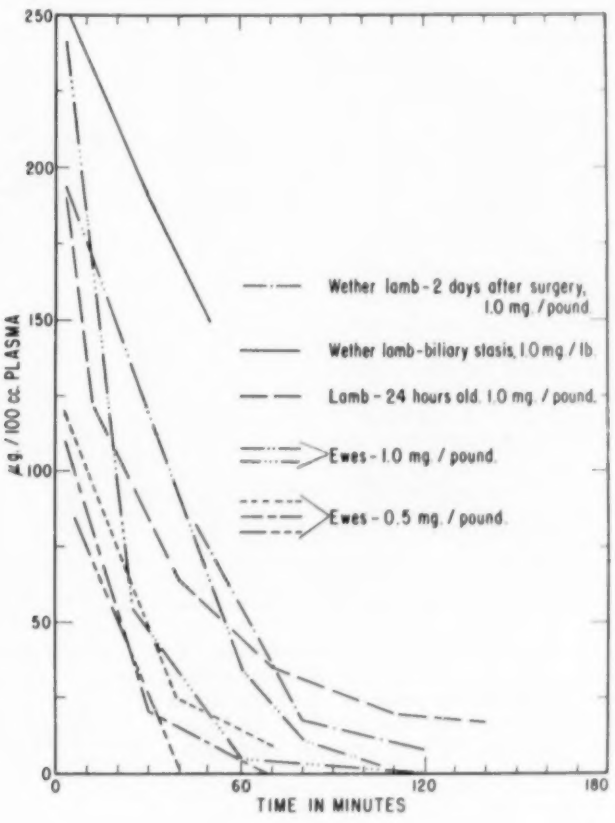
Because of the methods employed, it was possible to quantitate the recovery of intact hydrocortisone in the urine of all bovine animals and 4 ewes, in the bile of 1 wether, and in the urine and bile of 1 ewe (table 1).

There is ample evidence from previous reports and from the data above that the urinary route is not important in reducing the plasma titer of free or glucuronide-conjugated hydrocortisone in sheep and cattle. The more limited data on biliary excretion indicate that bile is not a major excretory pathway for the hormone in normal sheep.

TABLE 1—Recovery of Hydrocortisone in Urine and Bile of Sheep and Cattle

Animal	Dose of hydrocortisone (mg.)	Urine recovery (mg.)	Urine recovery (%)	Bile recovery (mg.)	Bile recovery (%)
Bull calf*	55	0.428	0.77	.....	.....
Heifer	175	0.594	0.34	.....	.....
Heifer	100	0.624	0.62	.....	.....
Cow	200	0.361	0.18	.....	.....
Bull calf**	100	0.317	0.31	.....	.....
Ewe	80	0.358	0.45	.....	.....
Ewe	40	0.138	0.34	.....	.....
Ewe	50	0.131	0.26	0.115	0.23
Wether	45	.....	.....	0.512	1.1
Ewe	80	0.368	0.46	.....	.....

* 24 hours old; ** 11 days old.



Graph 2—Hydrocortisone levels of ovine plasma after intravenous injection of hydrocortisone.

DISCUSSION

It should be reemphasized that only intact free and glucuronide-conjugated hydrocortisone were measured throughout this study. Consequently, the data presented can not be compared to those resulting from the use of isotopic techniques.

It is difficult to understand why sheep and cattle should eliminate hydrocortisone from plasma more rapidly than does man. The difference may be attributable to the adaptive metabolism of the hormone and to basic differences in metabolism between man and ruminant animals. It is of interest, and perhaps of importance, that hydrocortisone, so intimately associated with the process of gluconeogenesis, leaves the plasma most rapidly in ruminant animals in which endogenous gluconeogenesis is poorly developed and in which the pituitary-adrenal cortex axis is relatively weak.

Whereas it is relatively easy to explain slower hormone clearance in the wether by resorting to the over-all efficiency of the hepatic cells in this case, the difference in clearance as a function of age is not readily categorized. Slower disappearance of hydrocortisone from the plasma of newborn ruminants might be a function of immature hydrogenating mechanisms, less protein binding of the steroid, or low utilization rates.

Data on the quantitative urine recovery reported above correlate well with data of Nelson [2] who determined 17-hydroxycorticosteroids in arterial and renal venous blood after the intravenous infusion of cortisone into a dog, and found no evidence implicating the kidney as a major excretory organ for the hormone.

Although our limited data do not implicate bile as a major pathway for the excretion of hydrocortisone in normal sheep, Wynne [5] considered the bile to be the major excretory route of the steroid in moribund sheep. This observation might be correlated with data obtained by Sandberg and Eik-Nes [11] on dying human patients. These investigators presented evidence that the elevated plasma titer of hydrocortisone seen in such cases was caused by an impaired hydrogenation of the hormone. It is conceivable and even probable that the hydrogenation function of the ovine liver could be depressed *in extremis*, thus allowing abnormal biliary passage of hydrocortisone.

SUMMARY

Hydrocortisone was administered intravenously to normal cattle and sheep at dosage levels of 0.5 to 1.0 mg. per pound. Clearance of the plasma to normal levels occurred in 6 of 8 ruminating animals within one hour and in the remainder within two hours. The plasma titer of a newborn calf was still markedly elevated at 150 minutes; that of a newborn lamb, although elevated above normal levels at 150 minutes, was approximately half that of the calf. Biliary stasis and early liver fibrosis in a wether resulted in abnormal plasma retention of the steroid.

Recovery of hydrocortisone from urine of 9 animals was 0.18 to 0.77 per cent of the injected dose, indicating that bovine and ovine kidneys can not be considered to be major excretory organs for the elimination of hydrocortisone. Analysis of bile obtained via hepatic duct cannulation of 2 sheep gave a recovery of 0.23 and 1.1 per cent of the injected dose. The total recovery of hydrocortisone from both urine and bile in 1 ewe was 0.49 per cent.

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Isolation of a Pleuropneumonia-like Organism from Pigeons

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CHRONIC respiratory disease (CRD) of poultry is considered by many veterinarians to be caused by pleuropneumonia-like organisms (PPLO). Until recently, there has been no published work suggesting that pigeons are affected by the same or a similar disease organism. Winterfield [1] described a disease found in pigeons which was transmissible to turkey poults, producing sinusitis. He did not culture the causative agent. Van Rockel and Olesiuk [2] state that the pigeon is susceptible to the CRD agent, apparently basing this statement on Winterfield's work and on the following statement by Jungherr and Luginbuhl:[3]

Although the knowledge of air sac infection is in the early stages of development there are definite indications that the affection of broilers, which has given rise to the popular term, is etiologically and epiornithically related to chronic respiratory disease of chickens, and to sinus and air sac infection of turkeys, partridges, pheasants, and perhaps pigeons.

EXPERIMENTAL DATA

A pigeon fancier, having about 200 birds, was plagued with parasite problems in his loft. His birds were found to have *Tetrameres americana*, *Dispharynx spiralis*, *Capillaria columbae*, Ascaridia sp., Eimeria sp., *Hexamita columbae*, *Plasmodium relictum*,[4] and *Trichomonas gallinae*. Not all of these were present at any one time in any one bird. Serum from the birds was tested, through the courtesy of Dr. P. D. DeLay of the

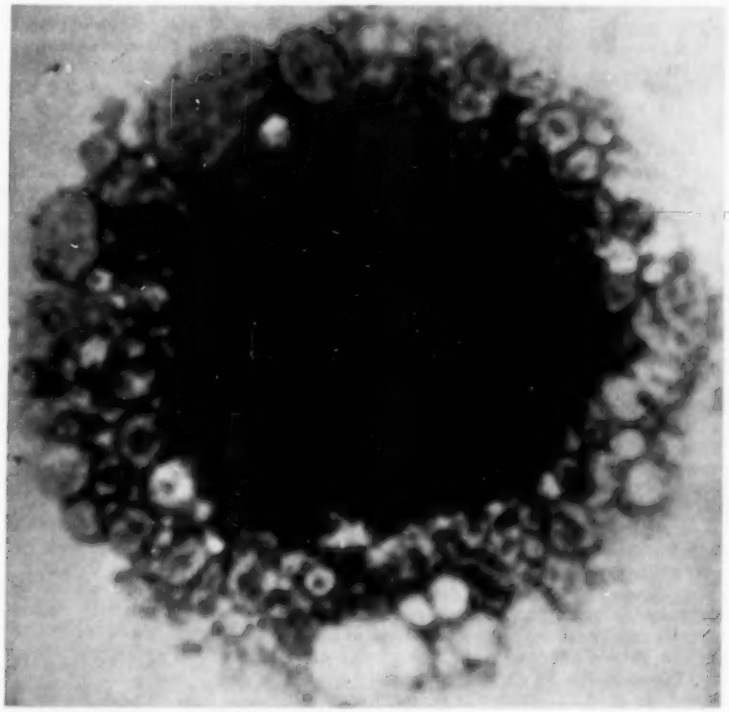

Fig. 1—Colony of pleuropneumonia-like organisms isolated from pigeon. Kleineberger-Nobel stain. x 1,000.

—Lorne Hardaker

Dr. Mathey is now employed by Pitman-Moore Company, Zionsville, Ind.

California State Department of Agriculture, for antibodies against *Salmonella typhimurium* with negative results. Attempts to culture Salmonella sp. from birds culled from the loft were unsuccessful. Apparently, *Trichomonas gallinae* was the chief cause of trouble. Since trichomonads are readily seen on microscopic examination of pharyngeal fluid samples, and since the various worm eggs may be identified in fecal samples, the owner requested that birds be checked before and after he treated them with antiparasitical remedies.

One such bird brought in for examination, a half-grown squab, was noticed to have considerable nasal exudate. Culture attempts were made, both by inoculating the exudate on tryptose horse blood agar plates (some to be incubated under 10 % CO_2 and some aerobically) and by using the method of Adler *et al.*[5] for PPLO. No growth was found on the blood agar plates. By Adler's method, colonies (on initial passage about 160 μ in diameter, on later passages, about 40 μ) were grown, which much resembled the PPLO colonies found in cultures from CRD of chickens. Similar colonies were obtained in cultures from other birds in the same loft and also from birds from a second loft.

Typical colonies were used as inoculum for 20 per cent horse serum broth and incubated for ten days. Profuse growth was obtained, as evidenced by turbidity, by heavy growth of PPLO agar plates inoculated with the broth, and by the large number of tiny coccoid bodies in stained preparations of the broth culture. An oily appearing film was noted on the surface of the broth. By centrifugation, a plate antigen was prepared from the broth culture, following the technique of Adler,[6] and tested against pigeon serums. No clear-cut positive agglutination was found.

A suspension of the organism was inoculated into the yolk sac of 6, 6-day-old chicken embryos. No deaths were noted in five days, at the end of which time yolk material was harvested and 0.2 cc. each was inoculated into 6, 6-day-old chicken embryos by the yolk sac route. One embryo died three days postinoculation. Yolk sac material from this embryo was then inoculated into 12, 6-day-old chicken embryos. One embryo died at 1 day of age, 2 died at 2 days, 3 died at 3 days, and 2 died at 4 days of age.

A suspension of the organism, from the third passage in penicillin broth, was inoculated into the following mediums (2 % serum fraction, phenol red indicator): dulcitol, salicin, maltose, mannite, sucrose, lactose, glucose. While there was profuse growth, as shown by turbidity, subcultures, and stained preparations, there was no fermentation in a nine-day observation. An oily appearing film was observed on the surface of the mediums. Similar results were obtained on repeating the inoculation on a second series of the mediums. A chicken CRD-PPLO culture fermented tubes of the same batch of the medium during the same period of observation.

Three pigeons approximately 3 months old were inoculated conjunctivally, intranasally, and in the palatine cleft with a culture of the organism grown for three days in the yolk sac of 6-day-old chicken embryos. In about six days, a slight, clear exudate was seen in the nares, and a mucoid exudate in the palatine cleft. The nasal exudate persisted for about one week, and the mucoid exudate in the cleft remained in evidence during the two months that the birds were held under observation. The serum of these birds at no time gave a clear-cut positive agglutination of the test antigen mentioned above.

Two 2-week-old chickens and 2, 6-week-old turkey poults were inoculated intranasally (0.2 cc.), conjunctivally (0.1 cc.), and intratracheally (1.0 cc.) with a three-day yolk sac culture of the organism in 6-day-old chicken embryos. The turkey poults were also inoculated intrasinusoidally (0.2 cc.). No symptoms were seen during a ten-day observation. At the end of the ten-day period, the birds were inoculated intramuscularly with 2 cc. each of the same culture which had been held in a refrigerator at about —8 C. After a week, the birds were bled and the serums tested against the plate antigen prepared from the broth culture. The two chicken serums agglutinated the antigen, while the two turkey serums did not. The chicken serums did not agglutinate an antigen prepared from a chicken CRD PPLO, nor did chicken CRD PPLO antiserum agglutinate the pigeon PPLO antigen.

SUMMARY

The isolation of a pleuropneumonia-like organism (PPLO) from a mild respiratory disease of pigeons is reported. The organism differs in fermentative ability, antigenicity, and pathogenicity for chickens and turkeys from strains of PPLO found in chickens and turkeys.

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Study of some Commercially Available Infectious Bronchitis Vaccines. I. Response of Chickens to Seven Infectious Bronchitis Vaccines

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SINCE THE virus of infectious bronchitis (IBV) of chickens was discovered in 1931 by Schalk and Hawn,[1] the gathering of basic information has been only sporadic.

The purpose of the work to be reported was to determine the response of birds to a number of IBV strains under controlled conditions to permit a study of the characteristics of infection by single strains. Attention was directed particularly toward the following aspects: (1) incubation period; (2) intensity of signs and morbidity in growing birds; (3) duration of signs; (4) communicability; (5) degree of immunity to challenge with a known pathogenic strain of IBV; (6) possible contamination of the vaccines with bacteria and other infectious agents.

MATERIALS AND METHODS

The chickens used throughout these experiments were White Leghorns, obtained as day-old chicks from two sources: (1) The Department of Poultry Husbandry, University of California, Berkeley [no known outbreak of infectious bronchitis (IB) was experienced in this flock for at least one year preceding the experimental trials]; (2) a commercial hatchery situated in an area heavily populated with poultry (Petaluma, California), where IB immune laying hens are prevalent. These chicks were defined as "commercial."

The birds, 4 weeks of age or older, used in the experiments were received in Davis as day-old chicks from either of these sources and raised under isolation at the University. Periodic testing prior to each experiment consistently showed them to lack detectable serum-neutralizing antibodies against IBV,[2] hemagglutination-inhibition antibodies against Newcastle disease virus (NDV), and agglutination antibodies against pleuropneumonia-like organisms (PPLO) and, therefore, irrespective of origin these chickens were considered susceptible to IB.

Virus Strains.—All of the strains of virus used, with the exception of strain 7, represented commercially prepared IB vaccines obtained directly from the manufacturers with the permission of the Department of Agriculture of the State of California. Upon arrival, the vaccines were stored in a refrigerator at 2 C. Each vaccine was tested within a month from date of arrival to avoid any deleterious effect of storage upon their activity. All of the commercial virus strains had been lyophilized and shipped in either hermetically sealed ampules or in small glass bottles.

The IBV strain labeled 7 was originally isolated from a field case by Dr. H. Van Roekel of the University of Massachusetts. Previous to its arrival in Davis in 1951, it had undergone 290 bird passages plus two chicken embryo passages. It was maintained in frozen allantoic fluid for six additional chicken embryo passages. Strain 7, inoculated intratracheally into susceptible chickens, caused pronounced respiratory signs and was used routinely as the challenge virus by the University of California at Davis.

With one exception, all manufacturers furnished a separate bottle containing a diluent. Both the diluent and the reconstituted vaccine were tested separately for bacterial contaminants, for PPLO, and for NDV. The lyophilized virus used in trial 5 was reconstituted with sterile 50 per cent glycerine in phosphate buffer solution (pH 7.4) as suggested by the manufacturer.

Isolation Facilities.—All of the experiments were conducted in a concrete building where chickens had never been raised. This building was well isolated and at least one mile from the nearest premises housing chickens. Each strain of IBV was tested separately and no other experiments with poultry were carried out in the building at any time. Every precaution was taken to ensure isolation and prevent contamination with other infectious agents. The preventive measures included complete change from street clothing to sterilized garments by the experimenter while in the building and showering when entering or leaving the building.

All equipment was sterilized both before and after each experiment. Whenever possible, a different unit was used for each strain studied. At the conclusion of each trial, each unit was thoroughly washed with hot water and disinfected with a 2 per cent sodium hydroxide solution. For eight days immediately preceding each experiment, known susceptible chickens were placed in the unit. These birds served as building and

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unit sentinels to detect the possible presence of IBV. In each case, the procedure of cleaning and disinfecting proved satisfactory since the birds never developed signs of IB during the period of exposure.

Testing Procedure.— All of the experimental birds were leg-banded and individual records kept. In each experiment, a significant number of birds was placed in separate cages in the same unit approximately 9 feet from the vaccinated birds. These served as room-exposure controls. No special precautions were taken in feeding and handling the controls.

Immediately prior to an experiment, blood samples were taken from representative birds of each age group for the serum-neutralization test for IB and for the hemagglutination-inhibition test for ND and for the PPLO agglutination test. Representative birds of each age group were removed to a separate experimental building and challenged with a virulent strain of IBV to ensure initial susceptibility of the birds to IB.

Immediately after reconstitution of the lyophilized vaccine in the diluent, the experimental birds were inoculated by the procedure prescribed by the manufacturer.

The chicks were examined at intervals of 24 hours or less to detect the earliest appearance of respiratory signs. Each bird was examined by holding its thorax against the observer's ear for possible presence of respiratory abnormalities. If the latter were not perceived, each bird was forced to expire by repeatedly applying a slight pressure on the thorax. In the absence of clearly audible respiratory sounds, each bird was permitted to flutter, by holding it by the tail several inches above the floor, to aggravate any signs of bronchitis.

Respiratory signs as a result of vaccination were classified as follows:

0 = no detectable signs.
+ = mild signs, consisting of coarse chirps and bronchial râles, detected by the procedure described above.
++ = moderate signs, consisting of moist bronchial râles and slight gurgling sounds.
+++ = severe signs, which included loud gurgling, sneezing, coughing, and gasping for air.

Each bird was also examined for presence of nasal exudate, watery eyes, and nervous disorders.

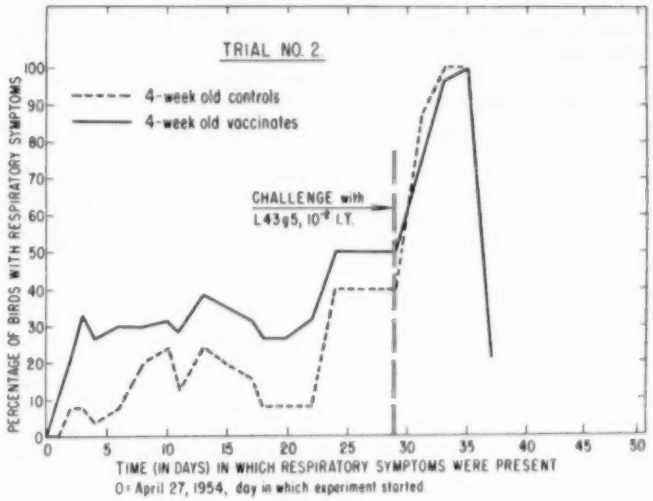
Immediately prior to challenge with strain 7, blood was taken from 10 to 100 per cent of the experimental birds for serum-neutralization and hemagglutination-inhibition tests.

TABLE 1—Response of Birds to Seven Strains of Infectious Bronchitis Virus

TRIAL NUMBER	EXPER. CHICKENS			Route of inocul.	Incubation period (days)	SYMPTOMS		Mortality	Spread ②	Immunity ③ (percent)	REMARKS
	No.	Age	Source			Intensity ①	Dur (days)				
1	50	1 day	"Commercial"	In Nasal	15	+ +	15	0	+	90	Prolonged incubation period. Rather severe vacc. reactions.
	50	22 days	Susceptible	" "	6-9	+ +	15	0	+	100	Suscept. chicks and older birds showed a more homogeneous reaction to vaccine.- Bacterial contamination of diluent
	26	10 wks	"Commercial"	" "	5	+ +	8	0	+	100	
2	50	4 wks	"Commercial"	In Musc.	1	0-+	29	0	?	0	Insignificant vaccination reactions. Immunity nonexistent or extremely poor.
	25	2 wks	"	" "	2	0-+	29	0	?	16	
3	85	7 days	Susceptible	In Nasal	2-6	+ +	25	0	+	74	Moderately severe and prolonged vaccination reactions. Immunity: Fair.- Bacterial contamination of diluent and vaccine.
4	40	5 days	Susceptible	In Nasal	15	0-+	11-32	0	±	54	Good immunogenic response only in the oldest birds.
	15	6 wks	"	" "	1-3	0-+	10	0	±	96	Vaccination reactions very mild in all age groups.
	40	1 day	"Commercial"	" "	15	0-+	16-18	0	±	61	Bacterial contamination of diluent and vaccine.
5	40	3 days	Susceptible	In Ocul.	3-9	+++	3-9	0	++	98	Marked spreading power. Severe vaccination reactions.
	40	3 days	"	In Nasal	3-11	+++	17	0	++	100	
	25	5 wks	"	" "	3-6	+++	8	0	++	100	Response to vaccination was not influenced by two different routes of inoculation (intra-nasal and intra-ocular).
	40	1 day	"Commercial"	" "	3-9	+++	11	0	++	100	
	40	1 day	"	In Ocul.	3-9	+++	3-9	0	++	98	
6	50	3 days	Susceptible	In Nasal	5-8	+ +	13	0	++	100	Vaccination reactions more severe in older birds but of shorter duration.
	50	5 wks	"Commercial"	" "	5	+++	8	0	++	100	Good immunogenic response.
7	80	7 days	"Commercial"	In Trach.	1 or less	+++	12-14	0	++	100	Incubation period very short.
	30	26 days	"	" "	1 or less	+++	12-14	0	++	100	Severe vaccination reactions.
	80	4 days	Susceptible	" "	1 or less	+++	12-14	0	++	100	Good immunogenic response.

① SYMPTOMS: - = coarse chirp, gurgling, sneezing, gasping, wet rales; 0 = no detectable symptoms; + = mild; ++ = moderate; +++ = severe
② To susceptible birds placed in same isolation unit. ③ Challenged intratracheally with 10,000 MID_{50} I.B.V.

The question marks under "spread" in vaccine 2 indicate that this characteristic could not be determined.


TRIAL NO. 2
4-week old controls
4-week old vaccinates
CHALLENGE with L43g5, 10⁻² L.T.
PERCENTAGE OF BIRDS WITH RESPIRATORY SYMPTOMS
TIME (IN DAYS) IN WHICH RESPIRATORY SYMPTOMS WERE PRESENT
0= April 27, 1954, day in which experiment started

Graph 1 — Morbidity of 4-week-old birds vaccinated intramuscularly with strain 2. Respiratory signs, as a result of vaccination, were mild and still present at time of challenge; following challenge they were severe and of short duration.

Usually on the twenty-ninth day following vaccination, or at a time when all of the vaccination reactions had subsided, all of the birds, including the controls, were challenged by intratracheal instillation of 0.20 ml. of strain 7 of IBV. This represented ca. 10,000 m.i.d.$_{50}$ for 6-week-old birds. Following challenge the birds were examined daily for 12 days for the presence of respiratory signs. The birds which did not show any signs were considered immune.

At the completion of each experiment, some or all of the birds were bled for the serum-neutralization and hemagglutination-inhibition tests and all the birds were killed. Necropsies were done on representative birds to detect lesions of other diseases.

RESULTS

The results are presented graphically (graphs 1 to 7) and tabulated (table 1).

Incubation Period.—The longest incubation period occurred in the day-old chicks vaccinated with strain 1 in which 15 days elapsed before 44 per cent of the birds showed definite signs of IB. Older birds (22 days), vaccinated with the same strain and at the same time, presented symptoms within four to eight days following inoculation. The shortest and most uniform incubation period was manifested by birds inoculated with the strains 5 and 7 of IBV. Strain 7 was the only one tested which caused severe respiratory signs in a high percentage (40 %) of birds in less than 24 hours. However, strain 7 was inoculated intratracheally and perhaps the route of inoculation may have had some influence on the incubation.

Intensity of Signs in Growing Birds.—An examination of the curves (graphs 1 to 7) indicates that all of the vaccines tested produced signs but of different degrees of severity.

Mild signs involving a very limited number of birds were observed in the groups vaccinated with strain 2. The signs became progressively more intense with strains 4 and 6. The 3-day-old birds inoculated with strain 6 reached a peak of signs on the eleventh day following vaccination as demonstrated by a 50 per cent incidence, while 76 per cent of the 5-week-old birds displayed moderately severe respiratory signs on the eighth day. In addition, 18 per cent of the 3-day-old birds displayed only mild respiratory sounds (+), and 4 per cent displayed no respiratory distress throughout the 30-day observation period. Only 2 per cent of the 5-week-old birds displayed mild reactions during the same period of observation.

The most severe respiratory signs were elicited by strains 5 and 7. Gurgling, audible at about 10 feet, sneezing, and coughing were pronounced in both trials. In spite of the severe respiratory signs, no significant mortality occurred among the birds inoculated with any of the strains tested.

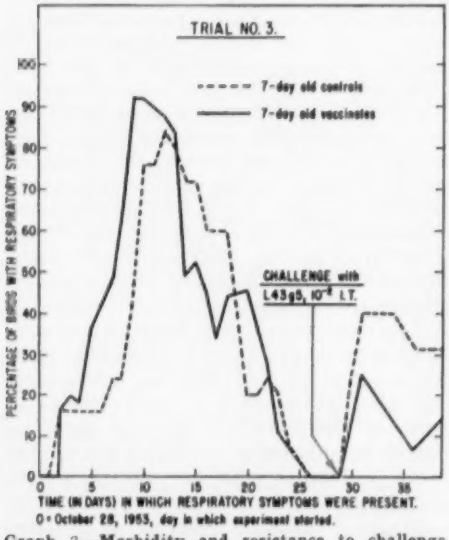
Strain 5 caused a reaction of a severe type (+++) in 96.0 to 97.5 per cent of the principals when the intranasal route was used. Swelling of the infraorbital

sinuses and nasal exudate were not observed in any of the experimental birds.

Duration of Signs.—The duration of signs was shortest with strain 6, in which severe respiratory signs persisted for one day in 28 per cent of the 3- and 5-day-old chicks, and for four or five days in the majority of the remaining 72 per cent (table 1). The respiratory signs persisted for 11 days in only 1 bird (3-day-old).

Strains 5 and 7 were also characterized by the short duration of signs which they induced. Respiratory signs in birds inoculated with these strains subsided between the eighth and the fifteenth day after signs first appeared. Duration of signs was longest in birds inoculated with strain 3. On the twenty-third day following inoculation with this strain, 12 per cent of the birds still manifested clinical signs of a respiratory disorder which finally disappeared on the twenty-sixth day.

Strain 4 produced the mildest respiratory reaction and, from a practical standpoint, the reactions probably would have gone unnoticed in the field. Only coarse chirps and bronchial râles, detectable solely by direct auscultation of the thorax, were recorded. The mild signs lasted for 19 days or more in day-old birds and 30 days or more in 5-day-old birds.



Graph 2—Morbidity and resistance to challenge of 7-day-old "susceptible" birds vaccinated intranasally with strain 3 of infectious bronchitis vaccine.

Strain 1 caused respiratory signs that were still present 15 days after inoculation but were no longer noticeable on the nineteenth day.

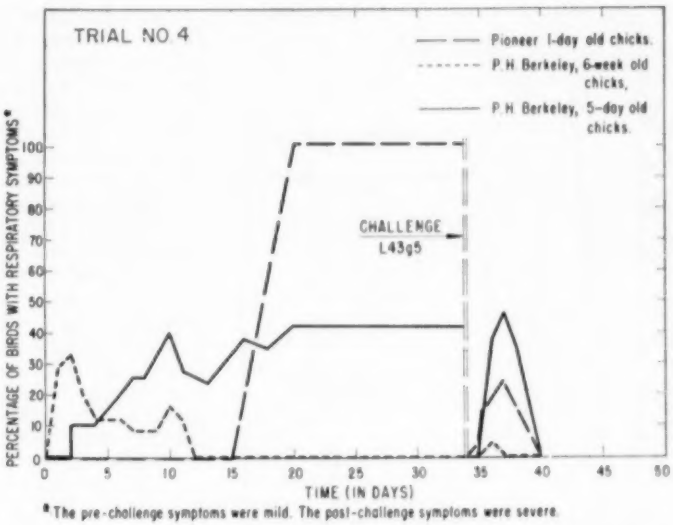
Communicability.—All of the IB vaccines tested spread to the controls within the same unit (table 1). Strain 4 presented the least spreading potential (1 of 10 birds was found to gurgle). The maximum degree of spread resulted with strain 5 among a group of ten 5-week-old birds. The room-exposure controls manifested clearly audible gurgling at the same time that the principals began to show signs and their intensity paralleled that observed in the vaccinated birds. The signs persisted for the same length of time since on the thirteenth day following exposure all of the 10 room-exposure controls were clinically negative. Further indication of spread to the controls was given by the fact that all of the birds resisted challenge with strain 7 of IBV, 27 days following exposure to the vaccine.

Strain 7 resembled strain 5 in its communicability. Twenty 7-day-old controls within a group of 80 inoculated birds of the same brood developed loud gurgling only four days following exposure to strain 7. While the first signs were noticed on the day following inoculation, all of the inoculated birds were clinically affected only 24 hours earlier than were all of the room-exposure controls. The severe respiratory signs (gurgling, sneezing, and coughing) subsided simultaneously in both the controls and the vaccinated birds. The spreading potential of strain 7 was confirmed by the solid immunity to IB 29 days later when challenged with the homologous strain.

The spreading potential of strain 6 was unique among the strains tested. This vaccine strain actually spread to 100 per cent of the room-exposure controls. At least 24 per cent of the controls failed to show any respiratory signs during the 30-day observation but they were immune to challenge later.

Strain 1 spread to 70 per cent of the room-exposure controls and strain 3 spread to 60 per cent.

Degree of Immunity to Challenge.—The data (table 1) show that strains 1, 6, and 7 gave 100 per cent protection against challenge with strain 7 in all of the age groups tested.


TRIAL NO 4
Pioneer 1-day old chicks.
P.H. Berkeley, 6-week old chicks,
P.H. Berkeley, 5-day old chicks.
CHALLENGE
L43g5
PERCENTAGE OF BIRDS WITH RESPIRATORY SYMPTOMS*
TIME (IN DAYS)
* The pre-challenge symptoms were mild. The post-challenge symptoms were severe.

Graph 3—Response of birds of different age groups (1 day, 5 days, and 6 weeks) to the intranasal inoculation of strain 4 of the infectious bronchitis vaccines. Note the different morbidity and resistance to challenge between the 6-week-old birds and the other two age groups.

The day-old and the 5-week-old birds vaccinated with strain 5 were completely protected against challenge with strain 7.

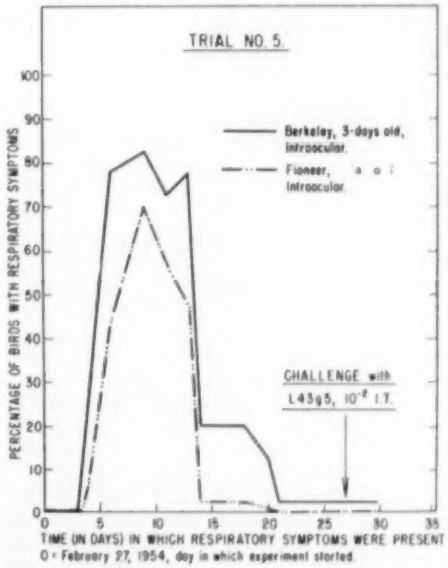
Strain 4 provided good protection to challenge only in 6-week-old birds (96% immune) while the degree of immunity, as judged by challenge, was poor in the day-old vaccinated birds (61% immune) and also in the 5-day-old vaccinated birds (54% immune).

Strain 3 stimulated immunity in 74 per cent of the 7-day-old principals.

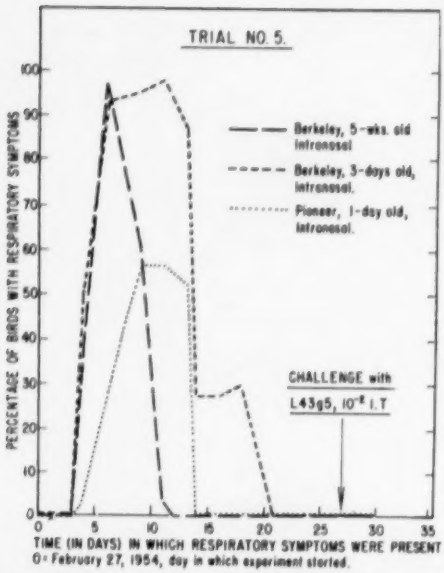
Contamination with Bacteria and other Infectious Agents.—Bacteriological examination of the different vaccines for presence of both aerobic and anaerobic bacteria revealed that four of the vaccines were contaminated and, in two instances, the diluent furnished by the manufacturer was also contaminated. These bacteria included Pseudomonas, hemolytic micrococci, Alcaligenes, Achromobacter, and *Escherichia coli*.

Discussion

During a survey to establish the extent of IB in California in 1951, 1952, and 1953, Zander and Sadler [4] found that approximately 50 per cent of the pullet flocks were immune to IB. Recapitulation and field surveys by the authors further established that IB occurred in poultry flocks in an apparently subclinical form since, in numerous instances, owners of carefully managed plants were unaware that an outbreak had occurred in their flocks. For these reasons, the program of establishing and evaluating the characteristics of viral strains employed in the commercially available IB vaccines is of importance to the poultry industry to pre-


TRIAL NO. 5.
Berkeley, 3-days old, intraocular.
Pioneer, intraocular.
CHALLENGE with
L43g5, 10⁻² I.T.
PERCENTAGE OF BIRDS WITH RESPIRATORY SYMPTOMS
TIME (IN DAYS) IN WHICH RESPIRATORY SYMPTOMS WERE PRESENT
0 = February 27, 1954, day in which experiment started.

Graph 4—Similarity in the morbidity and resistance to challenge of baby chicks of different sources (Berkeley and Pioneer) vaccinated intraocularly with strain 5 infectious bronchitis vaccine.


TRIAL NO. 5.
PERCENTAGE OF BIRDS WITH RESPIRATORY SYMPTOMS
Berkeley, 5-wks. old intranasal
Berkeley, 3-days old, intranasal.
Pioneer, 1-day old, intranasal.
CHALLENGE with L43g5, 10⁻² I.T
TIME (IN DAYS) IN WHICH RESPIRATORY SYMPTOMS WERE PRESENT
0= February 27, 1954, day in which experiment started.

Graph 5—Response of birds of different ages to the intranasal inoculation of strain 5 infectious bronchitis (IB) vaccine. Note the different degree of morbidity between the day-old chicks from IB-immune dams (Pioneer) and birds from susceptible dams (Berkeley).

vent the introduction of strains of IBV which could be more harmful than those already present in California.

A comparative study of seven vaccinal strains of IBV showed a great variation in their behavior in chickens of different ages and sources. Vaccine 2 was given intramuscularly because this was the only route directed by the manufacturer. Chickens of considerable variation in age were chosen to observe the influence of the age factor upon the response to the vaccine and, possibly, to have an estimate of the age at which chickens, vaccinated with a certain vaccine, would be expected to offer the best results. In some instances (vaccines 1, 4, and 5), day-old chicks were inoculated to study the possibility of using a certain vaccine at an earlier age than recommended by the manufacturer.

It is recognized that additional variations may be expected when these strains are applied to birds under the multiple environmental influences operating in the field. However, under the conditions of the experiments, individual and daily examination of each bird during the trials allowed evaluation of the effects of infection with several strains and revealed that the incubation period was shorter among older birds (vaccines 1, 4, 5, and 6). A shorter incubation period among older birds was observed in trial 1 in which day-old chicks obtained from an IB immune stock presented an incubation period of 15 days, whereas birds from the same breeding flock, after being raised 10 weeks under strict isolation, manifested an incubation period of only five days.

It was apparent that the age of the bird influenced the intensity of the respiratory signs. Generally, older birds reacted more intensively and with a higher morbidity than younger birds within the same experiment. Various explanations may be offered. This difference in behavior may have been due in part to the lack of passive immunity in the older birds (4 to 10 weeks) in contrast to the variable degree of such immunity in chicks (1 day to 3 weeks). Another explanation may be found in the opinion that the strains of IB, as seen today, have undergone a natural process of modification during the last 20 years so that their affinity gradually shifted from the chicks toward young adults, including pullets. A marked contrast in intensity of reactions of birds of comparable age was also apparent in trials 4 and 5. In trial 4, 5-day-old susceptible chicks presented very mild respiratory signs, if any, following inoculation while 3-day-old chicks from the same nonimmune breeding stock (trial 5) reacted with severe sneezing and gurgling. It is also of interest that within the same trials (4 and 5) birds from "susceptible" and "commercial" sources presented no apparent differences in their behavior toward the respective virus strains.

These data demonstrate that the intensity of reactions of birds toward a virus used in vaccine production is dependent not only on the age and source of the chickens, environmental factors, presence or absence of other diseases but also upon the characteristics of the strain employed.

Other differences in the characteristics of the various strains of the virus under study showed a variation in the duration of signs induced by the different strains; however, older birds appeared to recover sooner than the younger groups (trials 1, 4, 5, and 6).

Differences in the characteristics were also shown in the spreading potential of

the infection between inoculated and uninoculated chickens in the same room. All of the viruses spread to the exposure-controls, except strain 2. Strain 4 possessed the least spreading potential. Because of the exceedingly mild response of the birds to this particular strain, its spreading potential could be determined best on the basis of challenge 34 days following inoculation. The behavior of the birds to strain 6 differed considerably from the behavior of the chickens toward other strains. Only 30 per cent of the controls to strain 6 developed clearly audible respiratory signs and all of the controls were found to be completely resistant to a challenge dose of virus one month following initial exposure. These results indicate that strain 6 did spread to elicit an immune response without betraying its invasion of the host with clinical manifestations of an infection.

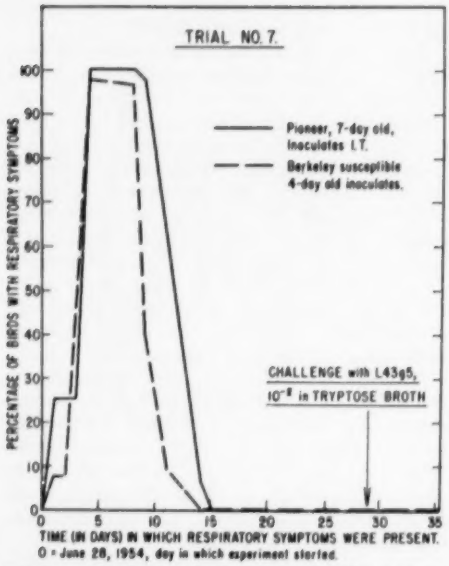
Only four vaccines of IBV tested (1, 5, 6, and 7) were endowed with a satisfactory immunogenic power (98–100%). Vaccine 4 provided good protection to challenge only among birds vaccinated at 6 weeks of age. Here again the age factor seemed decisive in establishing the degree of immunogenicity of a certain strain of IBV.

Immunity to a live virus vaccine is considered to depend upon substantial multiplication of a virus in the host tissues. Differences in the experimental birds and variations in the environment might account for variations in their immune response. However, the immunogenic characteristics of the strains studied were sufficiently marked to minimize the importance of the individual bird differences. In a limited number of field trials with the same vaccines, a good correlation was demonstrated between the experimental findings and the field observations.[5]

The birds used for these experiments were free of detectable PPLO as determined by cultural methods[6] and their serums were free of PPLO agglutinins.[7] Therefore, an opportunity was given to observe the effect of these IB vaccines on birds not debilitated by another interfering subclinical infection.

The conditions of strict isolation under which each strain of IBV was studied offered an opportunity to observe the behav-

Graph 6—Morbidity and resistance to challenge of 3-day-old "susceptible" chicks inoculated intranasally with strain 6 infectious bronchitis vaccine. Note that approximately 40 per cent of the inoculated birds were asymptomatic, but resistant to challenge with a strain of known pathogenicity (L43 g7).


TRIAL NO. 7.
Pioneer, 7-day old, inoculates I.T.
Berkeley susceptible 4-day old inoculates.
CHALLENGE with L43g5, 10⁻⁸ in TRYPTOSE BROTH
PERCENTAGE OF BIRDS WITH RESPIRATORY SYMPTOMS
TIME (IN DAYS) IN WHICH RESPIRATORY SYMPTOMS WERE PRESENT.
0 = June 28, 1954, day in which experiment started.

Graph 7—Similarity in response of birds of comparable age (4- and 7-day-old) but from different sources (Pioneer and Berkeley) to the intratracheal inoculation of strain 7 infectious bronchitis vaccine.

ior of uncomplicated bronchitis produced by the vaccine strains. Under these circumstances, there was not observed either swelling of the infraorbital sinuses or nasal exudate among apparently normal birds of various age groups and known origin.

The relative low pathogenicity and apparent immunogenicity observed among the strains tested, together with the occurrence of subclinical infection particularly with vaccine 6, indicates a proper trend in selecting a virus strain for use in an IB vaccine. However, search for a strain or strains having maximal immunogenicity with minimum pathogenicity should be diligently continued. Elimination of pathogenicity in a vaccine strain is indispensable to the successful control of this disease and it is reasonable to assume that an apathogenic strain of IBV may be less likely to trigger a latent infection.

SUMMARY

A comparative study of seven infectious bronchitis vaccines was performed under strict isolation conditions. An evaluation of the response of birds to the vaccines studied indicated that marked variations occurred when different infectious bronchitis vaccines were used. The incubation ranged from less than 24 hours (vaccine 7) to 15 days (vaccine 5). The intensity of signs varied from mild (vaccine 4) to severe (vaccine 1). The duration of signs varied from eight days (vaccine 6) to 32 days (vaccine 4) with considerable variations for the same vaccine. The degree of immunogenicity ranged from nonexistent (vaccine 2) to absolute (vaccines 1, 5, 6, and 7), but it was not necessarily related to the intensity of signs. While all the birds inoculated with vaccines 1, 5, and 7 reacted severely and were found solidly immune, only 58 per cent of the 3-day-old and 76 per cent of the 5-week-old birds responded to vaccine 6 with severe respiratory signs. Nevertheless, they were completely immune to challenge.

All strains of infectious bronchitis virus tested, with the exception of one, spread from the vaccinated birds to the susceptible exposure-controls. Dissemination appeared to be unrelated to the length of incubation and the duration of signs in the principals.

With the strains of virus employed, the age of birds at time of vaccination appeared to influence the incubation period of the disease. Within one experiment, older birds reacted sooner than younger birds. The intensity of the vaccinal reactions was more pronounced in older birds. The duration of the vaccinal response was shorter in older than in younger birds. A more satisfactory immune response was obtained in birds 4 weeks of age or older than in chicks 1 to 5 days of age.

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Electrophoretic Analyses of Serum from Chickens Infected with Newcastle Disease Virus

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RECENTLY, much attention has been devoted to the study of avian serum by means of paper electrophoresis. Most of the work, however, has been concerned with serum changes as influenced by endogenous and exogenous estrogens.[1, 9, 13] As far as the authors know, no work has been reported in which the relatively new paper electrophoresis method has been used to study changes of avian serum as influenced by viral antigens. The Tiselius electrophoresis method has been employed in several instances.

Sanders *et al.*[10] were perhaps the first to use the Tiselius method in their investigation of the serum of chickens affected with leukosis and described the presence of a new component in the serum. Dimopoullos[2] inoculated single comb White Leghorn cockerels with infectious bronchitis virus (IBV) and analyzed the serums electrophoretically. He noted a slow increase in the lethal dose$_{50}$ (l.d.) neutralizing indexes during the first and second weeks, followed by a period of marked increase with a maximum reached between the sixth and eighth weeks. No correlation was observed between the electrophoretic patterns and the changes in the l.d.$_{50}$ neutralizing indexes.

Schmittle[11] fractionated the serum from birds infected with the virus of Newcastle disease by means of cold ethanol and then analyzed the fractions electrophoretically. He obtained a pure *gamma* globulin fraction electrophoretically which he demonstrated to be rich in both hemagglutination-inhibition and serum-neutralization activities.

MATERIALS AND METHODS

Three-month-old White Rocks of both sexes, which had no previous exposure to Newcastle disease, were used. Four groups of 5 birds each were placed in cages in an isolated brooder house. Four birds in each cage were inoculated intranasally and intratracheally with 0.1 ml. of a 10^{-6} dilution of allantoic fluid which had titrated 10^{-8} in 6-week-old White Rock chicks. The fifth bird served as a control; when it developed the disease, spread of the virus was established. The virus used was the pathogenic GB or Boney strain. Blood samples were taken in the morning before feeding to minimize lipemia. Approximately 8 ml. of blood was taken from each bird by cardiac puncture, allowed to clot in sterile tubes, and the serum separated by centrifugation. Each bird was bled four times over a period of 28 days. The serum was kept frozen at −6 C. until analyzed.

A vertical-type electrophoretic chamber similar to the one described by Flynn and DeMayo[6] was constructed. It consisted of a piece of glass tubing 11 in. long and ⅞ in. in diameter, supported by vertically placed glass rods mounted in a narrow wood base. On each side of the wood base was a tank containing veronal buffer of approximately 1-liter capacity. Four strips of Whatman No. 3MM filter paper (1½ by 14 in.) were soaked with veronal buffer (pH 8.6, $\mu = 0.05$) on a glass plate, blotted with heavy filter paper, and draped carefully over the glass tube with the ends dipping in the buffer. The height of the glass tube was adjusted so that exactly 12 inches of the paper strips were exposed above the surface of the buffer. Then 20 cmm. of each sample from the same bird was streaked in a 1-inch band across the width of the strips at the point where it rested on the wide curvature of the supporting glass tube. A celluloid cover was placed over the whole apparatus and a potential of 380 volts with a current flow of 0.1 milliampere (ma.) was supplied from an adjustable power source. The current flow was maintained at 0.1 ma. during the first half hour of the run by adjustment of the power supply. At the end of eight hours, the current was turned off, and the rack holding the paper strips was immediately removed and placed horizontally in an oven preheated to 200 F. Drying, which was continued for ten minutes, was sufficient to completely dry the strips and fix the protein on the paper. After drying, the strips were immersed in bromphenol blue solution for ten minutes,[5] washed in 0.5 per cent acetic acid and dried at room temperature. Each strip was read in a densitometer (photovolt model 425) and the optical density vs. the distance of migration was plotted.

Total serum protein and albumin were determined photocolorimetrically by means of a modified biuret reagent.[8] Globulins were found indirectly by difference. Hemagglutination-inhibition tests were conducted according to Fabricant.[4]

RESULTS

A sample of normal serum and all the samples obtained from the same bird after

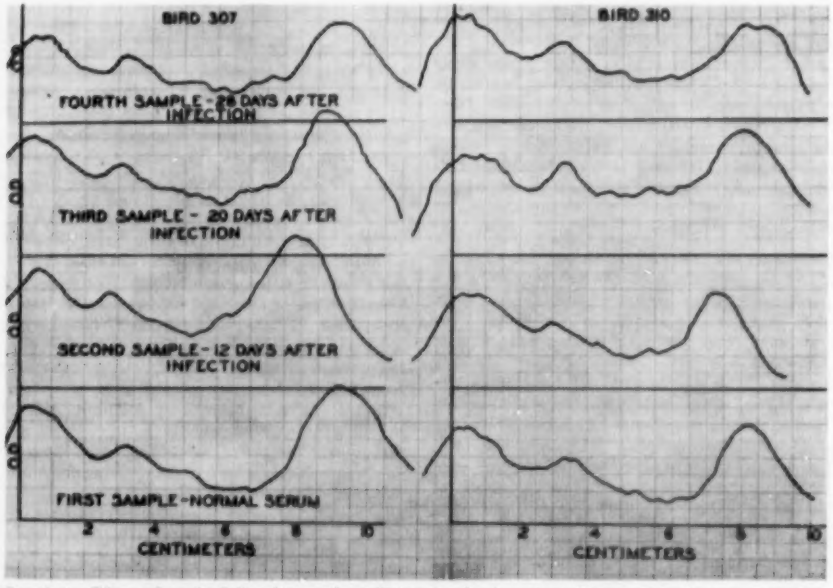
From the Department of Animal Pathology, Maine Agricultural Experiment Station, University of Maine, Orono. A portion from the thesis submitted by the senior author to the Graduate School, University of Maine, for a master of science degree in biochemistry. This investigation was made possible by a Fellowship from the Charles M. Cox Company, Boston, Mass.

infection were analyzed at the same time. The latter consisted of three samples obtained 12, 20, and 28 days after infection. In every case, the hemagglutination-inhibition test showed a maximum positive titer in the second and subsequent samples. Eight complete sets of four samples were obtained. In seven of these sets, a decrease in the distance of migration of the second serum sample was observed. The results obtained with two sets of serum samples are shown (graph 1). The decrease in distance as compared to the normal sample varied from a low of 0.50 cm. to a high of 1.60 cm. with an average decrease of 0.96 cm. for the seven samples. One sample did not show any change in the distance of migration. All eight sets of serums showed an increase in globulin in the second sample (table 1). The serum analyses showed an over-all hyperalbuminemia as compared to values for normal birds.[12]

The serum globulin was low except in the second sample which showed a marked increase. Electrophoretically, this hyperglobulinemia in the second sample was manifested by a decrease in the total distance of migration.

DISCUSSION

The significant changes, in the serum components observed in the experiment, were the marked rise in globulin and decreases in total migration observed in the second sample which was obtained 12 days after injection. A viremia is generally considered to occur in birds within one week after infection. In this study, virus isolation was not attempted from the blood any time after infection since viremia was not demonstrated by Karzon and Bang [8] and Hofstad [7] for longer than five days. This change in the second sample, therefore, was not considered to be due to virus particles. This condition might be due to the debilitation resulting from the infection and be similar to the globulin increase found by Dimopoullos,[2] who noticed an increase in the serums in birds infected with IBV during the first and second weeks. That this phenomenon may be due to antibody formation seems probable, since in some acute infections there is a sudden increase in antibody formation. This would not be detected with the usual hemagglutination-inhibition of serum-neutralization tests because, with these tests, the titer of



Graph 1—Plots of optical density against distance of migration of two sets of serum samples. In each case, notice the decrease in migration of the second sample.

TABLE 1—Comparison of Chemical Serum Analyses and Electrophoretic Migration Distances in Chickens Infected with Newcastle Disease Virus (GB Strain)

Bird	Sample	Albumin*	Globulin*	Total protein*	Distance of migration (cm.)
305	1	2.22	1.45	3.67	8.9
	2	2.37	2.18	4.55	7.9
	3	2.32	1.24	3.56	9.0
	4	2.27	0.93	3.20	9.6
306	1	1.96	0.78	2.74	10.2
	2	2.48	1.34	3.82	8.6
	3	2.48	0.72	3.20	9.7
	4	2.48	1.24	3.72	9.8
307	1	2.42	1.30	3.72	9.5
	2	2.78	1.40	4.18	8.2
	3	2.58	1.24	3.82	9.1
	4	2.63	1.24	3.87	9.2
310	1	2.94	1.34	4.28	8.4
	2	2.84	1.54	4.38	7.5
	3	2.89	0.75	3.82	8.3
	4	2.38	1.36	3.74	8.5
313	1	2.32	1.50	3.82	8.0
	2	2.53	1.65	4.28	7.4
	3	2.77	1.58	4.35	7.9
	4	2.78	1.81	4.59	8.3
319	1	2.22	1.60	3.82	7.5
	2	3.20	2.73	5.93	7.0
	3	2.84	2.01	4.85	7.8
	4	3.09	3.31	6.40	8.4
324	1	2.82	1.37	4.19	7.5
	2	2.55	1.64	4.19	6.6
	3	3.11	1.24	4.35	7.0
	4	2.20	0.62	2.82	7.6
311	1	2.74	1.39	4.13	8.0
	2	3.51	1.80	5.31	7.9
	3	2.66	1.02	3.68	8.5
	4	3.51	1.03	4.54	8.6

* Gm./100 ml. of serum.

the serum rises and remains at a high level over several months.

In this study, only 8 birds are reported in which a complete set of four serums was available. However, the same phenomenon was observed on 8 other birds in the same experiment, but due to mortality, laboratory accidents, and insufficient serums, four parallel samples were not available.

The original study was instituted as a possible aid in diagnosis of virus diseases of chickens. At the present time, the nature of chemical constituents of blood of birds at different ages is not completely known. This, together with very little published work on electrophoresis of avian blood, indicates that the technique should receive more research attention before it can be applied to diagnosis.

SUMMARY AND CONCLUSIONS

Blood serums from chickens infected with Newcastle disease were studied by means of filter-paper electrophoresis. The employment of a vertical-type chamber and strips of Whatman No. 3MM filter paper with a veronal buffer (pH 8.6 ionic strength 0.05) resulted in the separation of the serum into five components, *viz.*, *gamma* globulin, *beta* globulin, *alpha*-2 globulin, *alpha*-1 globulin, and albumin.

Serum taken from chickens 12 days after infection showed about a 20 per cent increase in globulin as determined by sodium sulfate precipitation. The only change noted electrophoretically for the same sample was a decreased distance of migration. No increase was discernible on the electropherogram of any globulin fraction.

Since individual variations, even among normal chickens, were frequently found, a study of isolated serum samples would appear to be of little value.

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Antigenic Studies Using Ammonium Sulfate
IV. The Sedimentation Effect of Ammonium Sulfate on *Salmonella Gallinarum*

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THE ANTIGENIC similarity of *Salmonella gallinarum* and *Salmonella pullorum*, causative organisms of fowl typhoid and pullorum disease, respectively, has been of academic interest for many years to workers in the field of poultry diseases throughout the world.

Kauffmann,[1,2] Edwards,[3] and Rodrigues and Pacheco,[4] among others, have attempted to separate the organisms on the basis of agglutination reactions and agglutinin absorption methods but have found them to be antigenically identical. Antigens prepared from either *S. pullorum* or *S. gallinarum* for use in the agglutination test are effective in detecting carriers of either organism.

On the basis of cultural, biochemical, and pathogenic characteristics, the two organisms are readily distinguishable and are considered by most investigators in the United States to represent two distinct types in spite of their close antigenic relationship. The existence of biochemical variants of both *S. pullorum* and *S. gallinarum* has been reported and, consequently, it has been suggested that there may be several biochemical subtypes in the *pullorum-gallinarum* group. The characteristics of the various subtypes have been summarized by Hinshaw.[5] Thumes, Naidu, and Noberega, as cited by Lilleengen,[6] have successfully distinguished *S. pullorum* and *S. gallinarum* through the use of bacteriophage capable of lysing one of the types but not the other.

In the Kauffmann-White schema, *S. gallinarum* and *S. pullorum* are assigned the antigenic numerals IX and XII. The XII antigen as it occurs in *S. pullorum* has been found by Edwards and Bruner[7] to be subject to a type of form variation, described earlier by Kauffmann,[8] and to be devisable into three partial antigens designated XII_1, XII_2, and XII_3. Those strains of *S. pullorum* with a preponderance of the XII_2 antigen are designated standard strains, while those in which the XII_2 antigen is well developed are referred to as variant strains. Since the XII_2 and XII_3 antigens may exist in a culture in widely varying proportions, a third antigenic type of *S. pullorum*, referred to as the intermediate type, has been described. The XII antigen of *S. gallinarum*, unlike that of *S. pullorum*, has not been found to exhibit form variation. The organism is described as existing in the XII_3 antigenic state and as being indistinguishable serologically from the standard type of *S. pullorum*. Serological studies indicating the absence of XII_3 antigenic forms in *S. gallinarum* cultures have been reported by Burr *et al.*,[9] Gwatkin and Dzenis,[10] Sudds and Jungherr,[11] and Marthedal.[12]

Antigenic studies of *S. pullorum*, using ammonium sulfate as reported by Williams[13,14] and Williams and Harris,[15] revealed that there is a distinct sedimentation effect of concentrated solutions of this salt on the various antigenic types of *S. pullorum* which can be correlated with the XII_2–XII_3 antigenic balance of the cultures. Standard antigenic types of *S. pullorum*, as well as cultures of *S. gallinarum*, were found to be readily sedimented by lower concentrations of the salt than were variant *S. pullorum* cultures. This finding further substantiates the differences, characteristic of the various antigenic types of *S. pullorum*, which are readily demonstrable by serological methods.

Previously, no attempt has been made to distinguish *S. gallinarum* and standard strains of *S. pullorum* using the ammonium sulfate technique, since the 330-Gm.-per-liter concentration of the salt used to type *S. pullorum* cultures has an identical sedimentation effect on suspensions of both *S. gallinarum* and standard strains of *S. pullorum*. Consequently, a more detailed antigenic study of *S. gallinarum*, using ammonium sulfate, was undertaken to determine if this technique might reveal antigenic differences in *S. gallinarum* and standard strains of *S. pullorum* that are not detectable by serological procedures. The results of this study are reported in this paper.

MATERIALS AND METHODS

Forty-one cultures of *S. gallinarum* and 23 standard type cultures of *S. pullorum* were examined. The *S. gallinarum* cultures were supplied by various laboratories in this country and Canada, and the *S. pullorum* cultures were obtained from the stock cultures maintained by this laboratory. The *S. gallinarum* cultures were identified as anaerogenic organisms which fermented both maltose and dulcitol. The *S. pullorum* cultures were aerogenic and fermented neither maltose nor dulcitol. All cultures of both *S. gallinarum* and *S. pullorum* included in this study were confirmed as XII_3 antigenic types.

Beef infusion broth cultures of each strain were incubated for 24 hours at 37 C. and then seeded into 300-cc. Erlenmeyer flasks containing 100 cc.

From the Animal Disease and Parasite Research Branch, U.S.D.A., A.R.S., Beltsville, Md.

of the K type agar of MacDonald.[16] Approximately 0.5 cc. of broth was used to seed the surface of each flask. The flasks were incubated for 96 hours at 37 C. Growth from each flask was harvested in 0.85 per cent saline solution containing 1 per cent formalin and phosphate buffer to provide a final pH of 6.4. The amount of buffer solution used to harvest each strain was varied, depending on the degree of growth. The dense suspensions thus obtained were further diluted with the buffer solution to provide a final density 100 times greater than tube 1 of the McFarland scale.

The ammonium sulfate solutions were prepared by depositing the desired quantity of analytical grade ammonium sulfate in a 1-liter volumetric flask and adding distilled water to adjust the volume to 1 liter. After the salt was completely dissolved, the solution was filtered through filter paper and stored in a tightly stoppered container.

A 0.5-cc. sample of each culture suspension was deposited in a serological tube (100 mm. by 13 mm.) and 8 cc. of the desired ammonium sulfate concentration was added, using a 10-cc. chemically clean pipette. The tubes were inverted several times to insure uniform mixing, following which they were incubated for five minutes in a serological water bath set at 55 C. The tubes were then observed for flocculation and immediately centrifuged for 15 minutes at 2,400 r.p.m. in a size 2 international centrifuge.

Final turbidity readings were made on the supernatant fluid of each tube and these readings were interpreted as the relative sedimentation effect of a particular concentration of ammonium sulfate on each culture suspension tested.

EXPERIMENTAL RESULTS

The relative sedimenting effect of varying concentrations of ammonium sulfate on suspensions of 41 cultures of *S. gallinarum* and 23 standard type cultures of *S. pullorum* is indicated (table 1). At a concentration below 250 Gm. per liter, *S. gallinarum* and standard strains of *S. pullorum* revealed identical reactions, *i.e.*, each type exhibited a markedly turbid supernatant fluid following incubation and centrifugation.

TABLE 1—The Relative Sedimentation Effect of Varying Concentrations of Ammonium Sulfate on Salmonella Gallinarum and Standard Type Salmonella Pullorum

	S. gallinarum	Standard type *S. pullorum*
$(NH_4)_2SO_4$ Gm./liter	Supernatant turbidity *	Supernatant turbidity
200	++	++
210	++	++
220	++	++
230	++	++
240	++	++
250	+	++
260	±	++
265	−	++
270	−	+
280	−	±
310	−	−
330	−	−

* − Clear supernatant; ± trace of turbidity; + moderate turbidity; ++ marked turbidity.

At a concentration of 265 Gm. per liter, all but two of the *S. gallinarum* suspensions exhibited a clear supernatant fluid indicative of complete sedimentation of cells. Two of the cultures exhibited a trace of turbidity at this concentration. All but seven of the *S. pullorum* suspensions maintained a markedly turbid supernatant fluid when exposed to 265-Gm.-per-liter salt concentration, indicating that these cultures were less susceptible than *S. gallinarum* to the sedimenting effect of this concentration of the salt. Seven of the 23 *S. pullorum* cultures studied revealed slightly less turbid supernatants than is considered characteristic for the type; however, none of these cultures showed the marked susceptibility of *S. gallinarum* to the sedimenting effect of the salt.

Standard strains of *S. pullorum* required an ammonium sulfate concentration of 310 Gm. per liter for supernatant clarification. Because of the narrow sedimentation range, flocculation results previous to centrifugation did not provide a constant basis for differentiation of antigenic types or indications of final sedimentation results.

DISCUSSION

The close antigenic similarity of *S. gallinarum* and standard strains of *S. pullorum*, discussed in an earlier section of this paper, is further borne out by the results of these studies. There is an indication, however, that minor antigenic differences may exist between *S. gallinarum* and standard strains of *S. pullorum*, since the former were consistently sedimented by lower concentrations of ammonium sulfate.

The range of sedimentation differences while relatively narrow, in contrast to that which characterizes standard and variant strains of *S. pullorum*, was distinct and characteristic for each type. The fact that two of the 41 cultures of *S. gallinarum* and seven of the 23 cultures of *S. pullorum* revealed a slightly different sedimentation pattern than that considered typical for each type suggests that overlapping antigenic subtypes may exist. These subtypes were not demonstrable by serological typ-

ing. The results of these studies indicate that there may exist minor antigenic differences between *S. gallinarum* and standard strains of *S. pullorum* which are not detectable by conventional serological methods.

SUMMARY

Suspensions of 41 strains of *Salmonella gallinarum* and 23 strains of standard type *Salmonella pullorum* were exposed to varying concentrations of ammonium sulfate, incubated for a short time and then centrifuged. It was found that an ammonium sulfate concentration of 265 Gm. per liter completely cleared the supernatant fluids of most *S. gallinarum* suspensions but had considerably less effect on the turbidity of suspensions of standard type cultures of *S. pullorum*. These observations suggest that there may exist minor antigenic differences between *S. gallinarum* and standard strains of *S. pullorum* that are not demonstrable by conventional serological methods.

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The Effect of Certain Antibiotics and Chemical Agents on Pleuropneumonia-like Organisms of Avian Origin

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PLEUROPNEUMONIA-LIKE organisms (PPLO) have been shown to be associated with chronic respiratory disease (CRD) of chickens and infectious sinusitis of turkeys by several investigators.[2, 9, 10, 12, 14, 19, 29] Considerable attention has been focused on the problem of eradication and control of these disease conditions because of the significant economic losses they have inflicted upon the poultry industry.[28]

The unsettled nature of this problem prompted further study of the effect of several chemical agents and newer antibiotics upon the PPLO associated with CRD of chickens using an *in ovo* method. In addition, an *in vitro* drug screening method was applied to several strains of PPLO of avian origin in order to obtain information on differences in susceptibility to antibiotic action and to correlate the *in vitro* and *in ovo* results.

MATERIALS AND METHODS

Cultures.—Two strains of PPLO, designated C and F, which were isolated from birds with separate field cases of CRD, were used in the *in ovo* drug trials. Infected yolk harvested from the seventh and thirty-first embryo passages of the F and C strains, respectively, was used for the inoculums for these studies. One-milliliter aliquots of the infectious material were stored in a dry ice chest and removed as needed during the experiment.

The C strain was much more resistant than the F strain to several antibiotics as determined in a preliminary *in vitro* trial. Consequently, the C strain was considered the resistant, and the F strain the sensitive strain.

Other PPLO strains from several avian sources were included in the *in vitro* trials. Fresh isolates were maintained in artificial culture as well as by chicken embryo passages. All strains were carried through several passages on artificial medium free of inhibitors prior to testing.

The PPLO strains used in this study qualified for inclusion in the pleuropneumonia group of microorganisms according to the criteria established by Sabin [26] with the exception that filaments were not observed in smears made from broth cultures. The organisms were typical for the PPLO associated with CRD and infectious sinusitis of turkeys as shown by their cultural, serological, and pathogenic properties.

Antibiotics and Chemical Agents.—The drugs which were tested were classified into four general types: (1) isonicotinyl hydrazones; (2) polyvalent arsenicals; (3) furan derivatives and; (4) antibiotics. The first group of drugs was selected on the basis of high antituberculosis activity [25] and with the thought that they would also be active against PPLO in a granulomatous process.[18] Several of the arsenicals were chosen based upon activity against PPLO infections in other animals.[1, 6, 21] The furan drugs were selected because of their wide antibacterial spectrum of activity.[4, 11] The drugs are listed in table 1.

TABLE 1—Antibiotics and Chemical Agents Tested for Activity Against Pleuropneumonia-like Organisms of Avian Origin

Isonicotinyl hydrazones*
5-chloro-salicylaldehyde isonicotinyl hydrazone
Salicylaldehyde isonicotinyl hydrazone
4-diethyl aminobenzaldehyde isonicotinyl hydrazone
Polyvalent organic arsenicals
N-acetyl-4-hydroxy-m-arsanilic acid (acetarsone,® Abbott Laboratories)
Phenarsone sulfoxylate (aldarsone,® Abbott Laboratories)
p-Ureidobenzene arsonic acid (carbarsone,® Eli Lilly & Co.)
Monosodium N-phenyl glycinamide-p-arsonate (tryparsamide,® Merck & Co., Inc.)
3-nitro-4-hydroxyphenyl arsonic acid (Dr. Salsbury's Laboratories)
4-nitrobenzene arsonic acid (Dr. Salsbury's Laboratories)
Furan derivatives
N-(5-nitro-2-furylidene-3-amino-2-oxazolidone (furazolidone, Dr. Hess & Clark, Inc.)
5-Nitro-2-furaldehyde semicarbasone (nitrofurazone, Dr. Hess & Clark, Inc.)
Antibiotics
Erythromycin (erythrocin lactobionate,® Abbott Laboratories)
Viridogrisein (Parke, Davis & Co.)
Oxytetracycline (terramycin,® Chas. Pfizer & Co., Inc.)
Chlortetracycline (aureomycin,® Lederle Lab. Div.)
Tetracycline (achromycin,® Lederle Lab. Div.)
Streptomycin sulphate (Merck & Co., Inc.)
Dihydrostreptomycin sulphate (Merck & Co., Inc.)
Chloramphenicol (chloromycetin,® Parke, Davis & Co.)

* Received through the courtesy of Dr. P. P. T. Sah and Dr. S. A. Peoples, Department of Pharmacology, University of California, Davis.

Each of the drugs in pure crystalline form was dispensed in 20- to 30-mg. lots in small screw-cap vials. Sterile distilled water was used as the diluent for the majority of the water-soluble drugs. One alcohol-soluble antibiotic, viridogrisein, was

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Appreciation is expressed to the companies indicated for providing the various antibiotics and chemical agents.

either dissolved in 10 per cent ethanol or suspended in egg yolk. The other water-insoluble drugs were suspended in either egg yolk or beef heart infusion broth (pH 7.8).

In Vitro Method.—The method used was similar to that described by Melen[20] and Robinson *et al.*[21] The test medium consisted of PPLO broth (Difco), pH 7.8, enriched with 20 per cent horse serum. The various dilutions of the antibiotics in broth were seeded with 0.05 ml. of a 48-hour culture of the PPLO strains tested. After incubation at 37 C. for 36 hours, a loopful of broth from each tube was streaked on PPLO agar enriched with 20 per cent horse serum. After the plates were incubated for 72 hours, they were examined for PPLO colonies. The absence of PPLO growth on serum agar was considered to be the lethal concentration of an antibiotic. The inhibitory concentration of an antibiotic was the dose that reduced the number of colonies by one half or more as compared with the untreated control.

Chicken Embryo Method.—The chicken embryos used in this study were obtained from a White Leghorn flock managed by this department. No symptoms suggestive of a respiratory disease outbreak were observed in this flock during the period the eggs were taken for this experiment.

The PPLO inoculum was inoculated into the yolk sac of embryos of seven days' preliminary incubation and the test drug was introduced into the allantoic cavity five to 24 hours later. Infected control eggs were handled similarly except that only the diluent used with the test drug was injected. Usually 10 embryos were used for each drug tested although as few as 5 were used in some of the preliminary trials. Generally, all trials were repeated one or more times depending on the information obtained.

Embryo deaths occurring within the first 48-hour period after inoculation of the PPLO were considered nonspecific and were excluded from the calculations. Previous experience had shown that embryos infected with PPLO of chicken origin did not succumb to the infection during this period. Although all trials were terminated on the thirteenth day after PPLO inoculation, embryos still living on this day were recorded, for convenience of calculation, as surviving an additional day.

The effectiveness of a drug was judged by its ability to prolong the mean day of death of the treated group beyond that of the control group. In addition, yolk from a representative number of embryos was cultured on agar medium to determine a drug's effectiveness in eliminating the PPLO from the chicken embryo. The data were statistically analyzed by first determining the analysis of the variance. Secondly, the variance of differences between means was estimated by $s^2_{\bar{x}} = \frac{(2)\cdot(s^2)}{n}$ where s^2 equaled the within-group mean square and n the number of embryos used

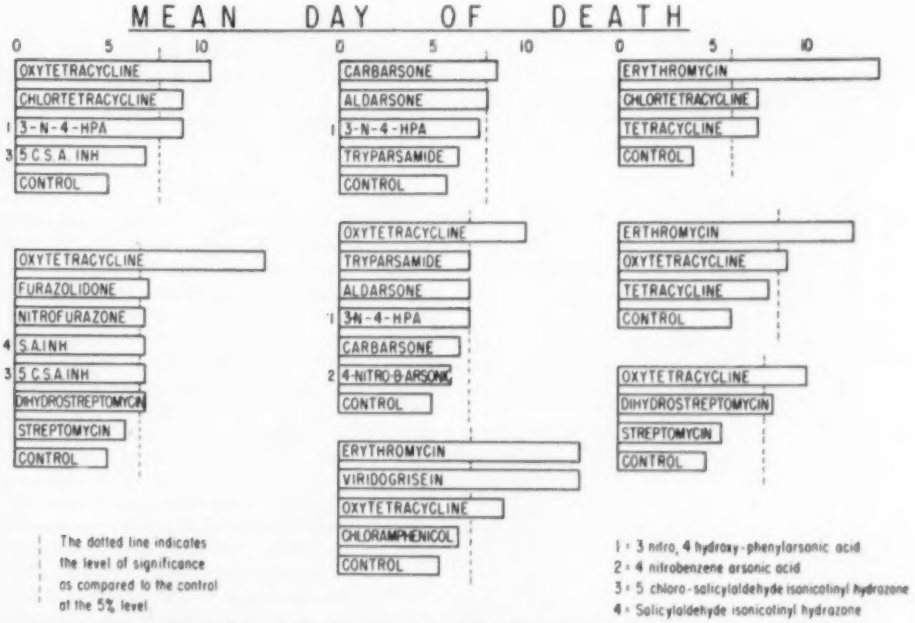


Chart 1—The effect of several antibiotics and chemical agents at the 1-mg. dose in protecting chicken embryos inoculated with the F strain PPLO (infectious inoculum of 10^4 to 10^5 I.d.$_{50}$ was inoculated five hours prior to treatment). Eight separate trials are illustrated.

per treated or control group. Lastly, the fiducial limits were determined to set the statistical difference that the treated group had to meet to be significantly different from the control at the 5 per cent level.[8, 27]

RESULTS

The arsenicals — aldarsone, carbarsone, tryparsamide, and 3-nitro-4-hydroxyphenyl arsonic acid—were not active against ten strains of PPLO under *in vitro* conditions. The results of testing several antibiotics under similar conditions are shown (table 2).

The effect of several chemical agents and antibiotics in prolonging the life of embryos inoculated with F strain PPLO is illustrated (chart 1). Since acetarsone was obtained late in the investigation, it was not included in the trials of other arsenicals shown in this figure. There was no significant prolongation of life of the embryos treated with it in three separate trials.

Several of the antibiotics which were effective in prolonging the life of embryos inoculated with the F strain were more critically evaluated against the resistant C strain. The effect of graded doses of these antibiotics in prolonging the life of embryos inoculated with this strain is illustrated (charts 2, 3, and 4).

Pleuropneumonia-like organisms were recovered on artificial medium from the yolk of the majority of the dead as well as destroyed embryos regardless of the strain of PPLO or drug used.

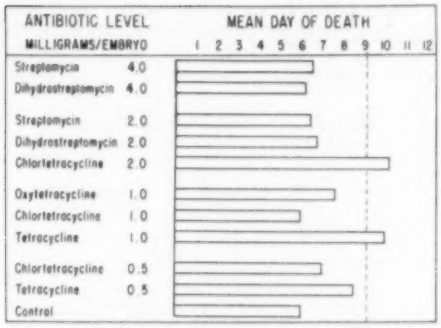
DISCUSSION

In Vitro.—The high *in vitro* activity of oxytetracycline as obtained in this investigation correlates well with the results of *in vitro* studies with PPLO of human origin.[18, 20, 22] Streptomycin was more active against the majority of the PPLO strains than dihydrostreptomycin, although the differences between the two antibiotics were not as marked as those reported for the PPLO isolated from man.[17] The *in vitro* studies indicated that differences in sensitivity do occur between strains isolated from different avian species and even among those isolated from a single species. The bacteriostatic nature of these antibiotics was evident from comparison of results of the inhibitory and the lethal concentrations, e.g., the lethal concentration of oxytetracycline for the D strain was more than 156 times greater than the inhibitory concentration.

TABLE 2—The In Vitro Study of the Lethal and Inhibitory Concentrations (μg./ml. of Medium) of Six Antibiotics Against Ten Strains of Pleuropneumonia-like Organisms of Avian Origin

Strains*	Oxytetracycline	Erythromycin	Tetracycline	Chlortetracycline	Streptomycin	Dihydrostreptomycin
T	1.56(<1.56)**	1.56(<1.56)	1.56(<1.56)	6.25(1.56)	12.5 (6.25)	6.25(>3.13)
P	>250.0 (25.0)	>250.0 (100.0)	>250.0 (6.25)	>250.0 (25.0)	500.0 (25.0)	>500.0 (50.0)
CT	50.0 (1.56)	1.56(<1.56)	12.5 (1.56)	>25.0 (12.5)	12.5 (>6.25)	>100.0 (>100.0)
F	3.13(1.56)	1.56(<1.56)	50.0 (6.25)	50.0 (6.25)	12.5 (>6.25)	12.5 (3.13)
D	>250.0 (1.56)	>250.0 (12.5)	>250.0 (1.56)	>250.0 (3.13)	150.0 (25.0)	>500.0 (50.0)
C	200.0 (1.56)	>250.0 (25.0)	50.0 (1.56)	>250.0 (12.5)	500.0 (12.5)	>500.0 (50.0)
H	>50.0 (1.56)	>50.0 (12.5)	>50.0 (1.56)	>50.0 (6.25)	12.5 (6.25)	25.0 (25.0)
J	50.0 (1.56)	50.0 (12.5)	150.0 (1.56)	50.0 (6.25)	>100.0 (25.0)	100.0 (25.0)
677	25.0 (1.56)	>50.0 (50.0)	50.0 (1.56)	50.0 (12.5)	>100.0 (12.5)	>100.0 (50.0)
G	>150.0 (1.56)	100.0 (12.5)	100.0 (1.56)	>100.0 (100.0)	6.25(<6.25)	25.0 (12.5)

* The T and P strains were isolated from the sinus exudates of a turkey and pigeon, respectively. The other strains were isolated from tracheal exudates of chickens.
** < signifies less than and > greater than the concentration listed. The numerals without parentheses indicate lethal and with parentheses inhibitory concentrations . . . see text for definition.

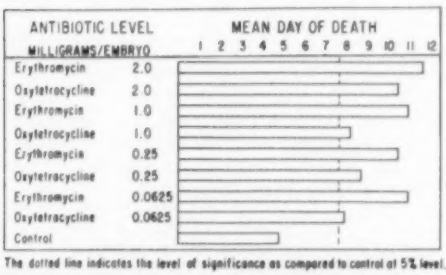


The dotted line indicates the level of significance as compared to control at 5% level.

Chart 2—The effect of graded doses of several antibiotics in protecting chicken embryos inoculated with the C strain PPLO (infectious inoculum of 10^7 l.d.$_{50}$ was inoculated five hours prior to treatment).

In Ovo.—Although the trend of *in ovo* activity of the commercially available antibiotics in these trials was similar to that reported by others,[7, 8, 13, 30] there were some differences that may be attributed partly to the method of evaluation and possibly also to differences in drug sensitivity of the various strains encountered.

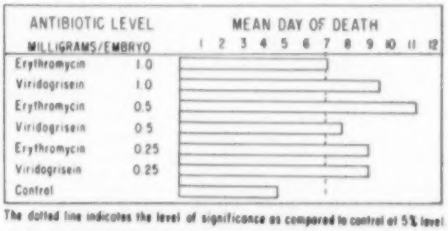
The *in ovo* results obtained with streptomycin and dihydrostreptomycin were not in agreement with the reports of others and in all trials both demonstrated an activity far below that of oxytetracycline when tested against either the sensitive F or the resistant C strain. Wong and James [30] placed streptomycin second in activity to oxytetracycline when tested against PPLO of avian origin. These investigators placed both the antibiotic and the PPLO into a single cavity, the yolk sac. Hoyt *et al.*[13] failed to protect embryos by simultaneously injecting streptomycin in the 2-mg. dose into the allantoic cavity and the PPLO into the yolk sac. Only 1 mg. was required, however, to destroy 10^8 l.d.$_{50}$ doses of PPLO when the injection of the antibiotic preceded that of the PPLO into the yolk sac.



The dotted line indicates the level of significance as compared to control at 5% level.

Chart 3—The effect of graded doses of erythromycin and oxytetracycline in protecting chicken embryos inoculated with the C strain PPLO (10^4 l.d.$_{50}$ of the infectious inoculum was inoculated five hours prior to treatment).

Except for the higher antibiotic doses of oxytetracycline or chlortetracycline required to suppress the C strain, the results with these antibiotics were in general agreement with those previously reported. The results obtained with oxytetracycline in this study would indicate that there is a wide range of antibiotic doses throughout which a slight variation in quantity would not appreciably influence treatment results, and that only at the higher doses would the antibiotic definitely be active. As seen in chart 3, the protection afforded at doses from 0.0625 to 1.0 mg. was not far beyond the statistically significant level.



The dotted line indicates the level of significance as compared to control at 5% level.

Chart 4—The effect of graded doses of erythromycin and viridogrisein in protecting chicken embryos inoculated with the C strain PPLO (infectious inoculum of 10^4 l.d.$_{50}$ was inoculated 24 hours prior to treatment).

This may account for the failure of the 1.0-mg. dose to protect the embryos inoculated with the C strain (chart 2).

Erythromycin was one of the more active antibiotics tested by the *in ovo* method. The ineffectiveness of this antibiotic against PPLO of human origin [16, 23, 28] would seem to indicate differences in sensitivity among strains of different host origin. This antibiotic was nearly as active at the lower dose of 0.0625 mg. as at the higher dose of 2.0 mg. against the resistant C strain.

Studies with viridogrisein were recently reported by Ehrlich *et al.*,[5] who found a high *in vitro* activity against the L-4 strain of PPLO of rodent origin. The *in ovo* results obtained in our study with viridogrisein against both the F and C strains indicated the same degree of activity as for erythromycin.

Although several of the antibiotics which

were tested in this study showed definite promise, none was found to be bactericidal under the *in ovo* conditions of the experiment.

SUMMARY

1) A method of evaluating drugs in the embryonating chicken egg was described; the infectious agent was inoculated into the yolk sac, the drug into the allantoic cavity.

2) *In vitro* antibiotic studies with several strains of pleuropneumonia-like organisms (PPLO) of avian origin revealed that tetracycline and oxytetracycline were the most active of the six antibiotics tested. Erythromycin, chlortetracycline, and streptomycin were of comparable activity but less active than tetracycline or oxytetracycline. Dihydrostreptomycin was least active.

3) The activity of several chemical agents tested against an antibiotic-sensitive strain of PPLO by the *in ovo* method was barely significant.

4) Of the antibiotics tested by the *in ovo* method against an antibiotic-sensitive and a resistant strain of PPLO, erythromycin and viridogrisein were the most active. The tetracycline drugs were comparable in activity but less active than erythromycin and viridogrisein. Streptomycin and dihydrostreptomycin were the least active antibiotics tested by the chicken embryo method.

5) Differences to antibiotic sensitivity were observed with PPLO of avian origin by both *in vitro* and *in ovo* methods.

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Antigenic Studies on the Psittacosis-Lymphogranuloma Venereum Group of Viruses. III. Detection of Ornithosis Hypersensitivity in Experimentally Infected Chickens

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IT HAS BEEN recently recognized that the occurrence of the ornithosis virus in chickens, turkeys, and ducks is widespread and that such fowl are responsible for a large number of cases in man.[1] With the increased number of recognizable human cases from sources other than psittacine birds, steps are needed to extend our knowledge of the reservoir hosts involved and to develop methods for the detection of the disease in these animals. The virus isolation procedure used for the definitive identification of the infection in chickens, turkeys, and ducks obviously does not lend itself to widespread epidemiological surveys. At the present time, the serological methods that have been developed have had a somewhat restricted acceptance. The serological methods are sensitive and specific but, from the standpoint of prohibitive costs and elaborate laboratory execution, they are not well suited for routine testing of large numbers of serums. The hemagglutinin test developed by Hilleman[2] is highly sensitive but still lacks common usage because of the lability of the hemagglutinin. The indirect complement-fixation test (c.f.) of Karrer *et al.*[3] has been used almost exclusively for revealing ornithosis exposure in those fowls whose serums do not fix complement. In view of the fastidiousness of the indirect c.f. test, only certain laboratories are willing to perform the test.

Our laboratory has been engaged in an investigation of the antigenic nature of the members of the psittacosis-lymphogranuloma venereum group of viruses and, during the course of the studies, a soluble antigenic complex was extracted from psittacosis virus strains.[4] The extract consisted of at least two c.f. antigenic fractions, and a skin test antigen capable of detecting hypersensitivity in chickens infected with ornithosis. When tested in chickens, this extract was more sensitive as an allergen than heated phenolized allantoic virus suspensions or than a commercial diagnostic complement-fixing antigen (Lederle). The opportunity was afforded, therefore, to study the relationship of hypersensitivity to infection, concomitant with the development of the intradermal method for the detection of ornithosis exposure in turkeys and chickens.

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MATERIALS AND METHODS

A brief description of the optimal conditions required for the preparation of the antigen is given below. Complete details are given elsewhere.[4] For production of the antigen, meningopneumonitis virus was as effective as other psittacosis strains and feline pneumonitis virus, and thus was used exclusively for this purpose. The virus was inoculated intra-allantoicly, the allantoic fluids harvested after four to five days of incubation, and the virus sedimented by ultracentrifugation. The sediments were resuspended in saline at pH 8.0 and 0.5 per cent sodium lauryl sulfate (duponol WA or lorol) added to give a final ratio of milligrams of detergent/milligrams of virus preparation nitrogen of 30:1. After 24 hours of extraction at room temperature, the extract was cleared of particles, and $BaCl_2$ was added to precipitate the detergent. The extract was cleared by centrifugation, and the resultant extract is referred to as crude antigen. Both c.f. and allergenic activities were precipitated from the crude extract by addition of acetic acid to pH 4.0 at 5 C., the recovered precipitates dissolved in phosphate buffer, and preserved with 1:10,000 merthiolate. Further purification was effected by repeated acid precipitation; however, for routine use, only a single precipitation with acid was required. The use of 0.5 per cent phenol as a preservative caused some inactivation of the allergen.

Single comb White Leghorn and Rhode Island Red hens and roosters were infected by the intramuscular route with 10^4–10^6 yolk sac l.d.$_{50}$ of psittacosis virus (Texas turkey strain). Skin testing was most satisfactorily performed by intradermal inoculation of the wattles with 0.05 ml. of the skin test reagents. Reactions (edema) were determined by measuring with vernier-scaled calipers the increase in thickness of the wattles in millimeters; however, reactions may also be judged by

palpitation. Measurements were made before and at various times following inoculation.

The indirect c.f. test was similar to the method given by Karrer *et al.*[5] In this test, complete hemolysis or no complement fixation (0) indicates a positive result. The titer is considered positive in the highest dilution in which fixation is completely inhibited (±).

RESULTS AND DISCUSSION

Edema occasionally appeared four to eight hours following intradermal inoculation of infected chickens but, in most cases, the height of the reactions was found 24 hours after inoculation. A 24-hour skin reaction in an infected chicken is shown (fig. 1). In lightly sensitized chickens, reactions faded rapidly and disappeared within 48 to 72 hours. The increase in wattle thickness of normal chickens was rarely greater than 0.3 mm.

Fig. 1—The 24-hour allergic reaction in a chicken infected with ornithosis.

TABLE 1—Comparison of the Detergent Extract with Other Antigens for the Detection of Allergy in Chickens Infected with Ornithosis

Chicken (No.)	Antigen: Crude detergent extract	Antigen: Killed allantoic fluid suspension	Antigen: "Soluble"** c.f. antigen
INFECTED THREE WEEKS			
52	0.0*	0.0	....
80	0.0	0.0	....
205	0.9	0.0	....
221	1.2	0.4	....
59	0.8	0.2	....
INFECTED FOUR WEEKS			
322	0.9	0.0	....
335	1.7	0.4	....
345	0.0	0.0	....
326	1.1	....	0.2
324	0.8	....	0.3
343	0.6	....	0.0
143	1.1	....	0.5
153	1.7	....	0.1
330	0.0	....	0.1

* Increase in wattle thickness, (mm.); 24-hour reaction.
** Lederle, complement-fixing antigen.

Presented in table 1 is a typical experiment comparing the crude extract, a high-titered heated phenolized complement-fixing antigen (allantoic fluid), and a "soluble" complement-fixing antigen (Lederle). For the early phase of the infection, the detergent antigen was more sensitive than the other antigens tested. Whether the sensitivity of these antigens is more comparable in chickens with older infections is as yet unknown. It is also shown (table 1) that some chickens, during the first four weeks of infection, failed to reveal allergic sensitization. However, only in rare instances were nonreactors found in groups infected for longer than five weeks. The relation of the indirect c.f. test and the allergic reaction in the acute and chronic phases of the infection is being studied.

TABLE 2—Specificity of the Intradermal Test for the Detection of Ornithosis Exposure in Chickens

	No. tested	No. positive	Positive (%)
Experimentally infected	142	131	92
Normal controls	84	3*	4

* 1 bird tested by indirect complement fixation test had a titer of 1:2.

Attesting to the specificity of the reaction of 84 normal control birds tested, 3 positive reactors were found (table 2). One of these birds was tested serologically and had a low indirect c.f. titer. Among groups in which sensitivity finally developed (longer than 5 weeks of infection),

TABLE 3—Stimulation of Allergic Reactions and Indirect Complement-Fixation Titers by Repeated Intradermal Inoculations of Normal Chickens with the Crude Skin Test Antigen

Chicken (No.)	Days since first skin test					
	0	6	18	24	34	43
70	0.0*(4,4,4)**	....	....	(3,4,4)	....	....
79	0.1(4,4,4)	....	....	(3,3,4)	....	....
76	0.1(4,4,4)	....	....	(2,3,4)	....	0.0(4,4,4)
72	0.0(4,4,4)	....	0.1	(2,3,4)	....	0.1(±,±,±,1)
77	0.3(4,4,4)	....	....	0.0(4,4,4)	....	0.2(1,1,2,4)
74	0.0(4,4,4)	....	....	0.0(3,±,±,3)	....	(4,4,4)
71	0.3(4,4,4)	0.0	0.0	(4,4,4)	....	(4,4,4)
73	0.0(3,4,4)	0.1	0.1	(4,4,4)	....	0.2(4,4,4)
78	0.2(4,4,4)	0.0	0.3	(2,2,3)	....	(2,2,2,4)
75	0.0(4,4,4)	0.1	0.0	(2,3,3)	....	0.2(3,3,3,4)
199	0.0	....	....	0.0	....	....
195	0.0	....	....	....	0.0	....
196	0.2	....	....	0.0	0.0	....
553	0.0	....	....	....	0.1	0.0

* Skin test reaction: increase in wattle thickness (mm.); 24-hr. reaction; ** indirect c.f. titer; doubling dilutions of serum beginning with 1:2 (± indicates complete inhibition of fixation).

131 (92%) of 142 birds tested gave distinct positive reactions (table 2).

Repeated skin testing of normal birds with the crude antigen failed to sensitize cutaneously (table 3) although questionable (birds 75, 78) or definite indirect c.f. titers (72, 77) developed after a single intradermal dose. In some cases (71, 73), repeated intradermal tests failed to elicit indirect c.f. titers. Chicken 74 developed an indirect c.f. titer of 1:8 24 days following a single intradermal test but failed to show a titer 19 days later. Repeated observations on the use of multiple skin test doses indicate that there is little danger of the development of allergic sensitivity resulting from the intradermal inoculation. Caution must be exercised, however, when serological studies are made subsequent to skin testing, and serums should be obtained prior to the skin test.

Precipitation of the complement-fixing activity from the crude extract with acetic acid was reported previously.[4] The allergenic activity was also precipitated by this procedure (table 4). Repeated acid

TABLE 4—Activity of the Crude and Partially Purified Skin Test Antigens in Chickens Infected with Ornithosis

Chicken (No.)	Antigen					
	Crude	Acid supernatant	No. of times precipitated with acid			
			1	3	4	5
			INFECTED			
566	0.8*	0	....	....	....	....
586	2.3	0.2	....	....	....	....
NT1	1.8	0	....	....	....	....
172	1.3	....	1.6	....	....	....
157	1.2	....	0.6	....	....	....
166	....	....	3.1	....	....	....
168	....	....	1.9	....	....	....
141	0.2	....	0.2	....	....	....
556	0.5	....	....	0.3	....	....
562	1.7	....	....	1.4	....	....
559	1.5	....	....	0.6	....	....
153	2.9	....	....	1.6	....	....
581	....	....	1.5	1.8	....	....
573	....	....	1.3	1.5	....	....
T	....	....	1.9	1.4	....	....
149	....	....	1.8	....	1.1	....
169	....	....	1.4	....	2.7	....
563	....	....	0.7	....	0.9	....
NT2	2.2	....	....	....	....	1.6
NT3	0.9	....	....	....	....	1.2
			CONTROLS			
553	0.0	....	....	0.0	....	....
196	0.1	....	....	0.0	....	....
195	0.0	....	....	0.1	....	....
452	0.1	....	0.0	....	....	....
453	0.0	....	0.0	....	....	....

* Increase in wattle thickness (mm.); 24-hour reaction.

precipitation failed to reveal any marked increase in activity of the allergen. Although allergenic activity was found associated with the complement-fixing antigenic fraction, preliminary experiments have indicated that two different antigens were involved.

It is felt that the skin test is promising and may prove to be a valuable tool for epidemiological studies in determining the extent of ornithosis infection in fowls. Such studies would only be intended to appraise the extent of ornithosis contact. Use of the skin test for diagnosis of the infection in individual birds should be discouraged, and such an act would indeed meet the same criticisms that have been levied against the use of the intradermal test for diagnosis of other infections in man and animals. Further evaluation of the skin test must await a more exhaustive study on the significance of the allergic test, and results of extensive field trial studies with naturally infected turkeys and chickens.

SUMMARY

1) The use of a soluble antigen for the detection of allergy in chickens infected with ornithosis was described.

2) When tested in chickens infected for three to four weeks, this antigen was more active as a skin test reagent than allantoic fluid viral suspensions and a commercial complement-fixing antigen.

3) Positive intradermal reactions were given by 131 of 142 experimentally infected chickens. Three supposedly noninfected chickens of 84 tested gave positive reactions.

4) Repeated intradermal inoculations in normal chickens stimulated the production of positive indirect complement-fixing titers but did not lead to a state of cutaneous hypersensitivity.

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Lysogeny in *Pasteurella Multocida*

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REVIEWS of lysogeny by Lwoff[1] and Jacob[2] have provoked considerable speculation as to the role of phage in nature. Investigations by Freeman[3] and Groman,[4] in which virulent lysogenic *Corynebacterium diphtheriae* were produced from avirulent strains by exposure to phage, presented an entirely new viewpoint as to possible epidemiological and pathological patterns in bacterial diseases. It is conceivable that other bacterial organisms may acquire (or lose) pathogenicity as they go from the nonlysogenic to the lysogenic state. With this in mind, *Pasteurella multocida* isolates were examined for the extent of lysogeny under natural conditions. Numerous animal diseases caused by this organism, particularly chronic fowl cholera, have nebulous characteristics. Noteworthy is the sudden and unexplainable appearance of the acute stage of the disease. This might possibly be due to a shift to the lysogenic state in the causative organism.

This report deals with an attempt to establish the extent of lysogeny among 25 *P. multocida* isolates from diseased fowl. A search of the literature revealed no clear-cut description of lysogeny in *P. multocida*, although the methods of isolating Pasteurella phages used by Rifkind and Pickett[5] indicated that possibly they may have been dealing with lysogenic organisms.

MATERIALS AND METHODS

The 25 isolates used in this study were obtained from Dr. T. A. Dorsey, South Dakota State College. The numerical designations of the cultures listed (table 1) are the same as those used by Dr. Dorsey in his studies of fowl cholera. Isolates were maintained on stock culture agar slants, but tryptose broth and agar were employed for all experiments.

A broth culture of each isolate was prepared and, after 48 hours of growth at 37 C., a sterile filtrate was obtained by Seitz filtration. To test for the presence of phage in each of these bacteria-free filtrates, it was necessary to find susceptible bacterial hosts. This was done by a trial and error method of testing each of the 25 filtrates against each of the bacterial isolates. The testing procedure consisted of incorporating approximately 10^8 actively growing bacterial cells into the thin top layer of each of a series of agar plates,[6] and microdrops of each filtrate were then spotted on the seeded surfaces of these petri plates; thus, five plates were adequate for checking the 25 filtrates against any one host. Plates were incubated at 37 C. and were periodically checked for growth and for clear zones at the spots where filtrates were added.

Inasmuch as clear zones are only presumptive tests for phage action, additional steps were taken to rule out antibiosis or nonspecific growth inhibition by nonphage substances that might be present in the filtrates. This was done by plating out serial dilutions of those filtrates that gave positive results in the presumptive test. If distinct plaques appeared, the presence of phage in the filtrates was confirmed. Antibiotics or other toxic substances do not produce plaques when adequately diluted and plated out.

In true lysogeny, phage exist inside bacteria in a prophage state through many generations of the bacterial host. It is also possible that phage may exist in bacterial cultures as a result of pseudolysogenesis,[7] *i.e.*, phage are mechanically carried on the surface of bacteria from transfer to transfer. The phage population is maintained by multiplication within susceptible bacterial cells that exist in the culture. To rule out pseudolysogenesis, single colonies from suspected lysogenic cultures were streaked. This procedure was repeated through three restreakings, which was sufficient to rid the culture of any mechanically carried phage. This restreaking process corresponds to diluting any carried phage to the point where it is improbable that any phage are present in the final culture.

RESULTS

The susceptible hosts and the isolates that harbored phage are shown in table 1. It may be seen that there was considerable range as to hosts that were lysed; therefore, not all of the lysogenic strains carried the same phage type. For example, filtrate No. 11 lysed hosts 6, 7, 16, 19, 23, 25, 27, and 28, but filtrate No. 2 lysed only hosts 7, 25, and 28. Thus, filtrates No. 11 and No. 2 did not contain the same phage types. Also (table 2), it may be seen that

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TABLE 1—The Extent of Lysogeny Among 25 Isolates of Pasteurella Multocida from Chickens with Fowl Cholera

Filtrate (No.)	Host cells																								
	1	2	3	3-1	4	5	6	7	9	11	12	14	15	16	18	19	21	22	23	25	26	27	28	29	30
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–
3	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	+	–	–	+	+	–	+	+	–	–
3-1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	+	–	–	–	+	–	+	+	–	–
5	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	–	–	–	+	–	+	+	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	+	–	–	+	+	–	+	+	–	–
11	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	+	–	–	+	+	–	+	+	–	–
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	+	–	–	–	+	–	+	+	–	–
15	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	+	+	–	–	+	–	–
16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	–	–	–	–	+	–	+	+	–	–
21	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	–	–	–	–	+	–	+	+	–	–
22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
26	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
27	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
28	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
29	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	+	–	–	–	+	–	+	+	–	–
30	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+ A zone of lysis when a microdrop of filtrate was placed on a petri plate containing a layer of host cells.

the two phage types produced different plaques, confirming the fact that the two phage types were different.

The filtrates tested comprised five distinct host range groups (table 1) and three plaque morphology groups (table 2). Filtrates 3, 9, and 11 constituted one host range group; 4, 5, 14, and 29, another; 19 and 21, another; 2, another; and 15, another. Filtrates that were alike as to host range were also alike as to plaque morphology with the exception of filtrate 5 which produced a distinct plaque morphology of its own.

It is conceivable that any one filtrate might have contained more than one phage type. However, the uniformity of plaques produced by each filtrate indicated that each was a pure strain. Additional evidence that each filtrate contained pure phage stock was furnished when new phage stocks were prepared from single plaques. These new phage stocks had the same host range properties as the original filtrates, thus indicating that each of the original filtrates contained but one phage type.

The extent of lysogeny among the 25 isolates is striking. Eleven (44 %) were positive for lysogeny (table 1). An even higher percentage might be anticipated if more host strains were available for susceptibility tests. Also, the tests for lysogeny were run under one set of conditions. It is possible that isolates that gave negative results for lysogeny might give positive results if temperature or some other factor were altered.

TABLE 2—Differences in Plaque Morphology Produced by Various Filtrates Plated on Host 28

Description of plaque	Filtrate
0.5 mm. diameter, irregular margin, clear	2,4,14,15,19,21,29
1.0 mm. diameter, irregular margin, cloudy	3,9,11
0.5 mm. diameter, irregular margin, very cloudy	5

SUMMARY

The extent of lysogeny among 25 *Pasteurella multocida* isolates was studied. All of these bacterial isolates were from chickens with fowl cholera. Eleven of the isolates were positive for lysogeny. Phages comprised five distinct groups as to their host range and three distinct groups on the basis of plaque morphology.

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Survival Time of *Mycobacterium Paratuberculosis*

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SURVIVAL time of *Mycobacterium paratuberculosis* has been observed under several conditions similar to those found in nature. Lovell *et al.*[1] have reported that the bacillus will survive approximately 270 days when stored in pond water, tap water, or distilled water, and 246 days in bovine feces. Vishnevskii *et al.*[2] reported survival in bovine urine for seven days and in bovine feces for 11 months.

This paper describes the effects of sunlight, freezing, drying, aqueous solutions of gelatin, dilute acid, dilute base, and bovine feces and urine on the viability of *M. paratuberculosis*.

PROCEDURE

Stock cultures of *M. paratuberculosis*, which were adapted to grow on a lymph gland medium described by Johnson,[3] were used. Since small laboratory animals can not be infected with *M. paratuberculosis*, it was necessary to rely on growth in cultures to determine viability of the bacillus after exposure. All inoculated cultures were incubated at 38 C. for 100 days and were observed periodically for evidence of growth.

Exposures were made under sterile conditions since the agents used in destroying contaminants also produce a harmful effect on *M. paratuberculosis*.

Experiment 1.—A total of 2.1 Gm. of *M. paratuberculosis* bacilli from representative cultures were divided into 25-mg. portions (dry weight) and placed in each of 84 screw-cap vials. These vials were divided into seven groups of 12 each and the following materials added to the tubes in each group:

Group 1—10 ml. tap water pH 7.0
Group 2—10 ml. tap water adjusted to pH 4.5
Group 3—10 ml. tap water adjusted to pH 8.5
Group 4—10 ml. tap water containing 1 per cent gelatin
Group 5—10 ml. saline (0.85% NaCl in distilled water)
Group 6—15 ml. bovine urine
Group 7—15 ml. mixture 9 parts bovine urine plus 1 part bovine feces.

The vials were maintained at 38 C. in darkness. At intervals of 30 to 90 days, one of the 12 vials in each of the seven groups was removed. The contents of each vial was centrifuged, the supernatant decanted, and the sediment inoculated onto solid medium in six culture tubes. The tubes were examined at 30-day intervals for 90 days to determine if the bacilli were viable (table 1).

TABLE 1—Survival Time of Mycobacterium Paratuberculosis Maintained Under Various Conditions

Treatment	Inoculation intervals (months)							
	1	4	6	9	11	14	17	19
Saline	+	+	+	+	+	+	+	N
Tap water pH 7.0	+	+	+	+	+	+	+	N
Tap water pH 5.0	+	+	+	+	+	+	N	....
Tap water pH 8.5	+	+	+	+	+	+	N	....
1% gelatin in tap water	+	+	+	+	+	+	N	....
Bovine urine	N	....	....	....	....	....	....	....
90% bovine urine, 10% feces	N	....	....	....	....	....	....	....

+—growth observed; N—no growth observed.

Experiment 2.—To study the effect of drying, 1.6 Gm. (dry weight) of *M. paratuberculosis* obtained from stock cultures was distributed in 200-mg. quantities in each of eight glass tubes (14 mm. by 140 mm.). The bacilli in one tube were immediately inoculated onto medium for use as a control. The remaining seven tubes were plugged with cotton, loosely capped with tin foil, and placed in a desiccator over H_2SO_4 at room temperature. Seven days later when the bacilli had become dry and crumbly, three tubes were removed from the desiccator and placed in a darkened container at 38 C. At intervals of 60 days, bacilli from a tube from each of the two environments were inoculated on solid medium and incubated at 38 C. These cultures were observed at 30-day intervals for three months. This procedure was continued until the supply of stored bacilli was exhausted, which was 17 months for the material maintained in darkened container at 38 C. and 47 months for the material maintained in the desiccator. Viable bacilli were observed in all cultures.

Experiment 3.—Two screw-cap vials each containing 250 mg. (dry weight) of *M. paratuberculosis* bacilli were each maintained under a variety of conditions as follows. Tube 1 was in a cold storage freezer at −14 C. for five months, in the refrigerator at 4 C. for five months, and in the incubator at 38 C. for eight months. Tube 2 was stored in the freezer at −14 C. for 12 months and in the refrigerator at 4 C. for five months. Bacilli from both tubes were still viable after such exposure as demonstrated by culture.

Experiment 4.—Quantities of bacilli (100 mg.) were placed in a 1/16-in. layer on pieces of filter paper. Each piece of paper was placed in a Petri

From the Regional Animal Disease Research Laboratory, Agricultural Research Service, U.S.D.A., Auburn, Ala.

dish. The dishes were each covered by a piece of cellophane 0.038 mm. thick taped at the edge to prevent contamination of the bacilli. These containers were placed in the sun each day and the highest temperature in an identical container was recorded along with the hours of direct exposure to the sun. Temperatures in the container ranged as high as 44 C. during exposure. Block *et al.*[4] show that cellophane 0.03 mm. in thickness is almost equal to pure quartz glass for ultraviolet ray transmission. The bacilli were viable after 65 hours of such exposure but not after 100 hours of exposure.

TABLE 2—Growth of Mycobacterium Paratuberculosis on Medium Containing Bovine Urine, Feces, or a Mixture of Both

	pH of medium	Growth compared to normal as 100
1. Control medium	6.7	100
2. Medium containing 1% urine, 1% feces	6.8	95
3. Medium containing 2.5% urine, 2.5% feces	6.4	10
4. Medium containing 5% urine, 5% feces	6.7	5
5. Medium containing 10% urine, 10% feces	6.4	0
6. Medium containing 2% urine	6.3	100
7. Medium containing 5% urine	6.3	20
8. Medium containing 10% urine	6.6	2
9. Medium containing 20% urine	7.0	0
10. Medium containing 2% feces	7.0	75
11. Medium containing 5% feces	6.5	75
12. Medium containing 10% feces	6.7	70
13. Medium containing 20% feces	6.8	5

Experiment 5.—Three liters of lymph gland medium were prepared and divided into 13 equal portions. Portion 1 was used as a control while the 12 remaining portions were each mixed with varying percentages of bovine urine, bovine feces, or a mixture of both (table 2). The pH of each medium was recorded and all vials were inoculated with *M. paratuberculosis*. They were incubated at 38 C. and observed for evidence of growth.

It will be observed from these results that the rate of growth was inversely proportional to the amount of bovine urine in the medium. Although the addition of bovine feces to the medium also inhibited growth, the results were not nearly as marked as in the case of the urine.

DISCUSSION

From the experiments described in this paper, it is evident that *M. paratuberculosis* is very resistant to many of the conditions found in nature. It is more resistant than the tubercule bacillus since Smith[5] found that tubercule bacilli suspended in water only survived five months in darkness at room temperature and 14 months in darkness at 45 F. There were no marked differences in survival time of bacilli maintained in either solutions of dilute base or dilute acid compared with tap water at pH 7.0. This is of interest since soils may range in pH from above neutral to slightly acid. The bacillus is only moderately resistant to sunlight. It is very resistant if maintained free of feces and urine in a darkened area. In nature, such a site might be a clump of trees where cattle stand for long periods during warm weather. Bacilli shed in such an area could be washed free of feces and urine by rain and, at the same time, not be exposed to direct sunlight. As a control measure for Johne's disease, it would appear advisable to eliminate such areas entirely or replace them with moveable structures if shade is needed. Moving such structures at intervals would insure exposing all areas of pasture land to direct sunlight.

SUMMARY

Mycobacterium paratuberculosis was exposed to various conditions and survival time determined by culturing the exposed bacilli. Survival time in bovine urine and a mixture of bovine urine and feces was less than 30 days. Survival time in tap water or saline was between 17 and 19 months. In tap water adjusted to pH 5.0 or pH 8.5 or containing 1 per cent gelatin, survival time was between 14 and 17 months.

Bacilli remained viable when maintained in a desiccator over H_2SO_4 for 47 months. Other bacilli which were exposed similarly for seven days, then removed and maintained at 38 C. in darkness for 17 months, were still viable.

Bacilli stored first at −14 C. for five months, then 4 C. for five months, and finally 38 C. for eight months were still viable. Bacilli stored at −14 C. for 12 months and then at 4 C. for five months were also viable as demonstrated by culturing.

Mycobacterium paratuberculosis placed on filter paper and exposed to sunlight through cellophane was viable after 65 hours of exposure but not after 100 hours of exposure.

The growth of *M. paratuberculosis* was inhibited in proportion to the quantity of

bovine urine and feces added to a standard culture medium.

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A Simple Device for Carrying Multiple Pieces of Tissue Through Reagents from the Fixative into the Paraffin Block

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IN SMALL histology laboratories, it is often not economically feasible to own an automatic machine that will carry numerous pieces of tissues from the fixing reagents, through the dehydrators, clearing reagents, melted paraffins, and finally into the block. The method described below has been used

Fig. 1—Tissue baskets in 8-ounce specimen jars.

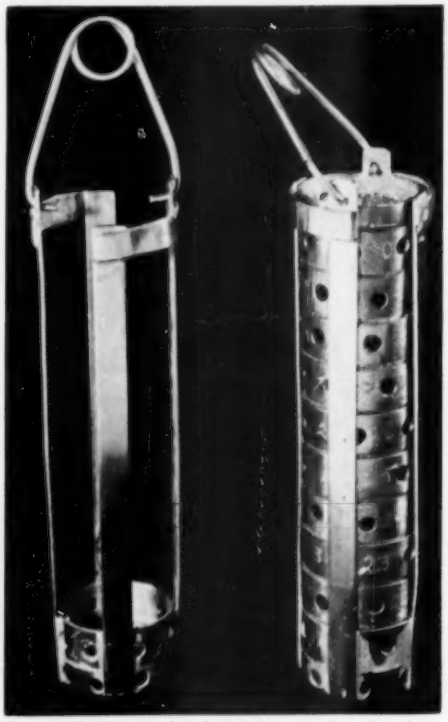

Fig. 3—Left, completed stainless steel rack; right, rack and tissue baskets in place.

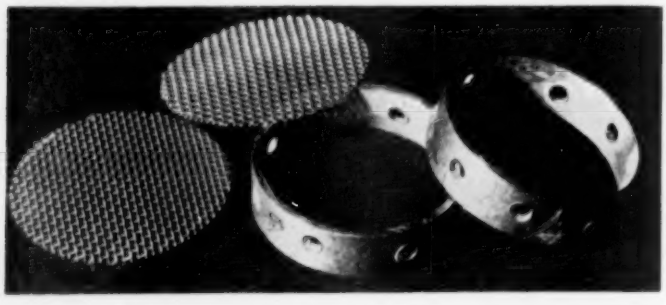

Fig. 2—Stainless steel rings (1½ in.) and screens.

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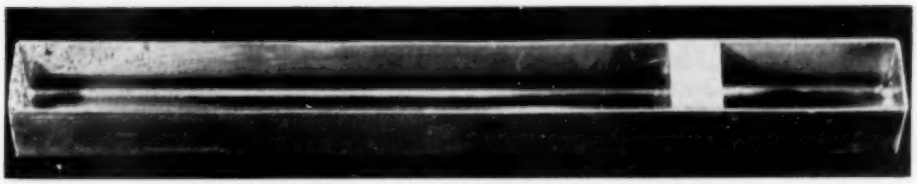

Fig. 4—Paraffin tray with adjustable brass block in place.

satisfactorily for two years in the histology laboratories in the Veterinary Anatomy Department at Iowa State College. It is especially useful when multiple pieces of similar tissue are taken from the same animal. It is also a time saver and yet accurate identification of the tissue samples is not jeopardized. In addition, smaller quantities of reagents can be utilized with equally good results (fig. 1).

This device is made entirely of stainless steel. A special stainless steel soldering flux is used with ordinary tin and lead solder. The device (fig. 3) consists of a

Specimen No._____ Fixative Used:_____ Date:_____

Breed:_____ Sex:_____ Notes:_____

Dog No.	Tissue	Block No.	Age	Date Autopsied	Pieces	Capsule

Date	Time	Hours	Reagents

Fig. 5—Data sheet.

series of rings cut from either 1-in. or 1½-in. 14-gauge stainless steel tubing (fig. 2). Holes 3/16 of an inch in diameter are drilled in the wall of the rings at either ¼-in. or ½-in. intervals. The top ring which forms the lid is ⅛ of an inch in height, whereas the bottom ring is ¼ of an inch high. The intervening rings which will form the tissue baskets are ⅜ of an inch in height (fig. 2). The tissue baskets and lid are held in series by a rack. The four upright posts of the rack (fig. 3) are also made of 14-gauge stainless steel straps ¼ of an inch wide and long enough to accommodate ten tissue baskets. The four upright posts, spaced equally, are soldered to the bottom ring and the first tissue basket (fig. 3). Alternate pairs of adjacent posts are fastened together at the top with a piece of strap shaped to fit the contour of the tissue baskets. This allows free access to the baskets when removing them from the rack. These top braces are soldered to the upright posts just under the lid of the rack. Two of the projecting posts are cut off flush with these braces on the top. The remaining two are left longer in order to accommodate a 3/16-in. hole which is drilled in each of them to receive the ends of the spring handle. The spring handle holds the lid tightly against the top basket (fig. 3). The spring handle is made from a ⅛-in. stainless steel welding rod which is bent one and one-half times around a ⅜-in. bolt.

A tray (1 in. by 1 in.) variable in length, fashioned from 12-gauge stainless sheet steel, is used as the form for the paraffin tissue block (fig. 4). A movable brass block (1 by 1 by ⅝ in.) is fitted into the tray thus permitting blocks of variable lengths to be poured. The tissues are taken from the baskets and placed in the tray filled with melted paraffin in the position corresponding to their number in the rack. The paraffin, upon cooling, shrinks enough to allow the block to pull free from the tray. Identifying labels can be placed over each tissue in the long block. The blocks can be stored in this convenient form indefinitely. A simple form can be printed to record time, dates, reagents, etc., used with each set of tissue baskets (fig. 5).

It is possible, by this method, to embed a large number of different tissues accurately, economically, and at a great saving of time.

Actinobacillosis. I. An Evaluation of Cultural Characteristics of Selected Strains of *Actinobacillus Lignieresi*

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In 1902, Lignières and Spitz[5,9] described a granulomatous disease of cattle in Argentina which in the years immediately preceding had caused serious losses. The organism associated with the characteristic lesions was subsequently designated *Actinobacillus lignieresi* and the disease actinobacillosis. Isolation of this organism from cattle and other animals has been reported by workers throughout the world.[3-6,10,13,15-17,19,20] Moreover, the occurrence of actinobacillus infection in man has been reported.[1,18]

During the past months, five strains of *A. lignieresi*, resembling those described in the literature,[4,14,16,21] were isolated in Florida from characteristic granulomatous lesions of the head and cervical region of cattle. Since discrepancies have been reported regarding the biochemical, pathogenic, and antigenic characteristics of this organism,[21] reinvestigation of this problem was indicated.

This paper presents results of bacteriological, biochemical, and pathogenic studies using the Florida strains along with nine cultures recovered from cattle and sheep in different sections of the United States and England. In addition, the sensitivity of these strains to certain antibiotics and chemotherapeutic agents was investigated.

A history of the disease in Florida cattle indicated that introduction of an animal with clinical actinobacillosis into noninfected herds resulted in the occurrence of a number of clinical cases of the disease within a few months. There was a correlation between the clinical stage of development of the granulomas and their consistency (fig. 1), suggesting a tendency toward hard tumor formation in the older lesions. Bone lesions were not encountered in any of the infected animals. All attempts have failed to recover actinomycetes or organisms other than actinobacillus from the lesions of the naturally occurring disease.

Circumstantial evidence of a human infection was obtained in the case of a 14-year-old girl who had been exposed to infected cattle. The case came to our attention after a suppurative metastatic abscess of the cervical region had responded to antibiotic treatment. Blood serum of this patient agglutinated five Florida bovine actinobacillus antigens in dilutions of 1:160 and 1:320, while ten control human serums revealed no demonstrable agglutinins or reacted in low titers of 1:10 and 1:20.

Materials and Methods

The origin of the strains studied are presented in table 1. Isolation of the organism from cattle

TABLE 1—Sources of Cultures of Actinobacillus Lignieresi

Strain	Species of origin	Source and date of isolation
FM	Bovine	J. W. Williams—Clewiston, Fla., 1954
G	Bovine	M. Ristic—Ft. Myers, Fla., 1954
N	Bovine	M. Ristic—Orlando, Fla., 1954
90	Bovine	M. Ristic—Lakeland, Fla., 1954
Dwarf	Bovine	M. Ristic—Gainesville, Fla., 1954
10811	Bovine	American Type Culture Collection, 1933*
4222	Ovine	J. M. Coffey—N. Y. State Dept. Health, 1941 **
1185	Ovine	E. A. Tunnicliff—Montana Vet. Res. Lab., 1951
32774	Bovine	N. Y. State Dept. Health, 1932*
33443	Bovine	N. Y. State Dept. Health, 1933*
208	Bovine	E. O. King—Communicable Disease Center, U.S.P.H.S.†
209	Bovine	Same as strain 208
210	Bovine	Same as strain 208
211	Bovine	Same as strain 208

* Originally isolated by L. Thompson, Minnesota.
** Originally isolated by E. A. Tunnicliff, Montana.
† Originally isolated in England; date of isolation unknown.

Acknowledgments are due to the above mentioned investigators who generously donated cultures for this study.

From the Departments of Veterinary Science (Sanders, head; Ristic, associate pathologist) and Bacteriology (Herzberg, assistant professor), University of Florida. Florida Agricultural Experiment Station journal series No. 466. Dr. Williams is a pathologist, Morrell Memorial Hospital, Lakeland, Fla.

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with actinobacillosis in Florida was accomplished by: (1) searing the surface of the excised tissue, (2) macerating tissue pieces in a Ten-Broeck grinder, (3) adding approximately an equal volume of sterile saline, and (4) plating the resulting homogenate. For primary isolation and subcultures, blood agar and brain-heart infusion plates (agar 1.8 %) were used.

The method used to observe colonial variation consisted of examining 24-hour cultures plated on thin Albimi agar* preparations to which 0.5 per cent plain agar had been added to increase solidity of the medium. Examination was performed at magnification of 10 to 20 x as viewed using oblique, indirect transmitted light, according to the method of Henry.[7] To study biochemical characteristics, cultures were grown on blood agar for 24 hours at 37 C. The resultant growth was washed from the agar surface with sterile 0.85 per cent sodium chloride solution, standardized to a density of McFarland standard tube 3 and 0.1 ml. inoculated into the following test mediums in duplicate: carbohydrates in bromthymol blue broth base and phenol red broth base, tryptone broth, nitrate broth, urea broth, litmus milk, and gelatin. All tests were carried out as described in the Manual of Methods for Pure Culture Study of Bacteria.[12]

Five bulls 6 to 9 months old, as well as guinea pigs, rabbits and hamsters, were used to study pathogenicity. For this purpose, 16- to 20-hour growth of the organisms was washed from Albimi agar with sterile saline, filtered through sterile glass wool, and standardized to a preferred density. Groups of five 11-day embryonating chicken eggs were inoculated via the chorioallantoic cavity with 0.2 ml. of undiluted cell saline suspension and tenfold dilutions to 10^{-7}. A viable plate count of the last two highest dilutions was made. The eggs were candled daily for four days following inoculation and all deaths recorded. The chorioallantoic fluid of the dead embryos was immediately cultured on Albimi agar plates and incubated for 24 hours at 37 C. after which time they were examined. On the fifth day following inoculation, all eggs were opened and cultured as described.

Determinations of the *in vitro* sensitivity of *A. lignieresi* to antibiotics and sulfonamides were made using the paper diffusion disc and plate method. Suspensions of the test organisms were streaked heavily over the surface of the blood agar plate. The inoculated plates were incubated for one hour at 37 C., to allow the inoculum to become absorbed into the surface of the medium, then standard bactosensitivity discs (Difco) of three concentrations were applied to the surface. Only one substance was tested on each plate in order to avoid possible synergistic or antagonistic activities. The zones of inhibition were measured after 20 hours of incubation at 37 C.

* Obtained from Albimi Laboratories, Brooklyn 2, N. Y.

RESULTS

Colonial Variations.—Observation of various strains by oblique transmitted light revealed the presence of the following three colonial types:

a) fluorescent, 5.0–10.0 mm. diameter, flat, golden red, fluorescence, roughened surface, often with irregular edge (fig. 2);

b) granular, 2.0–4.0 mm. diameter, bluish, raised, entire edge, translucent with fine internal granulation (fig. 2);

c) dwarf, 0.5–1.0 mm. diameter, raised gray colorless, edge entire.

The fluorescent type predominated in

Fig. 1—Granulomatous lesions of the cervical region excised from cattle naturally infected with Actinobacillosis. Necrotic center surrounded by fibrous tissue is evident.

stock cultures. The granular type tended to dissociate to fluorescent; hence, it was maintained by periodic selection and subculture. Granular cultures firmly adhered to the surface of the solid medium and required scraping in preparing suspensions. The dwarf type occurred only in freshly isolated cultures. A tendency toward dissociation of the dwarf type to granular and fluorescent forms was observed upon second or third subculture, particularly in broth. No morphological differences were observed among the cells of the various colonial types. The cells appeared as gram-negative, short, rounded-end bacilli, approximately 0.5 to 1.0 μ in length. Pleomorphic and coccal forms, as well as long bacillary forms, were occasionally present.

Biochemical Reactions.—The results are presented in table 2. There were no noticeable differences in reactions of the fluorescent and granular colonial types. Acid was produced in dextrose, galactose, and maltose. All strains produced acid from sucrose, xylose, mannose, and fructose, with the exception of the dwarf strain on fructose and the A.T.C.C.† strain 10811 which failed to attack all four sugars. Variations among the strains were observed in their activity on arabinose and dextrin. An influence of the incubating temperature on such reactions was noted. Other carbohydrates studied were not attacked by any of the strains. Reduction of nitrate to nitrite and production of urease was characteristic of all strains with the exception of strain 10811, which failed to exhibit this reaction. All strains were indol negative and produced only traces or no H_2S, while strain 10811 was indol positive and produced H_2S. The optimal growth temperature of the Florida strains, the English strains, and strain 32774 was 37 C. Strain 10811 grew well at temperatures from 18 to 37 C., while the other strains grew equally well at temperatures from 25 to 40 C. All strains were catalase positive and did not attack gelatin and litmus milk.

Pathogenicity.—Two bulls inoculated subcutaneously, on either side of the neck, with 5.00 ml. of saline suspension (4–5 x 10^8 cells/ml.) of freshly isolated strain FM developed clinical granulomas at the site of injection three months following inoculation. Pure cultures of Actinobacillus were secured from cervical lesions of these animals. During the fourth month after injection, 1 of these animals developed severe respiratory difficulties characterized by labored breathing, nasal and lacrimal discharge, and died in approximately one week. On the surface of the lungs of this animal were numerous minute, shotlike,

† American type culture collection.

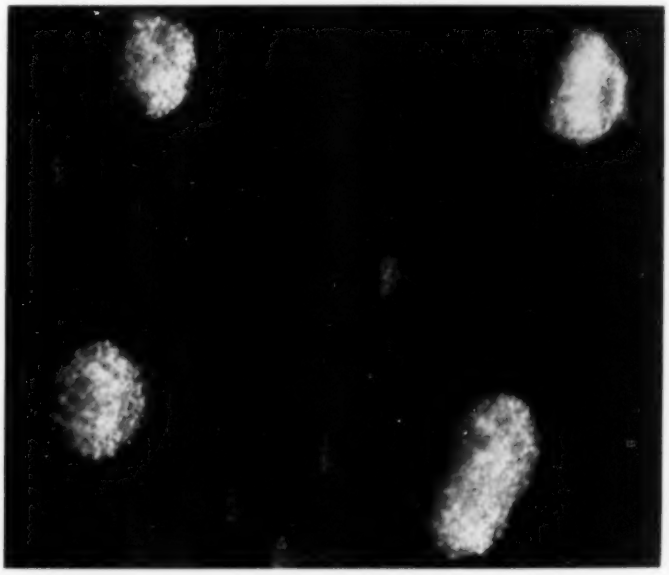

Fig. 2—Colonies of Actinobacillus lignieresi, strain FM, as viewed by oblique transmitted light. Large colonies are the fluorescent variant; small colonies are the granular variant. Approximately x 20.

TABLE 2—Biochemical Reactions of Actinobacillus Lignieresi

	Strain numbers													
	G	N	FM	90	1185	4222	32774	33443	10811 A.T.-C.C.	Dwarf	208	209	210	211
Arabinose	—	+30 —37	+30 —37	—	+	—	—	—	—	—	—	—	—	—
Dextrin	+30 —37	+30 —37	+30 —37	+ —	—	—	—	+	+	—	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fructose	+	+	+	+	+	+	+	+	—	—	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inulin	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lactose	—	+	+	+	+	+	—	—	—	+30 —37	—	—	—	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	—	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	—	+	+	+	+	+
Melezitose	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Raffinose	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Rhamnose	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Salicin	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sorbitol	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sucrose	+	+	+	+	+	+	+	+	—	+	+	+	+	+
Trehalose	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Xylose	+	+	+	+	+	+	+	+	—	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	—	+	+	+	+	+
Indol production	—	—	—	—	—	—	—	—	Weak	—	—	—	—	—
Urease	+	+	+	+	+	+	+	+	—	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatinase	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Litmus milk changes	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H_2S production	T	T	T	T	—	—	T	—	+	T	T	T	T	T

+ Positive reactions; in the case of sugars, acid and no gas; — no reaction; T trace.

yellowish white nodules (fig. 3). Upon sectioning the lung, a number of similar areas was observed throughout its substance. Pure cultures of Actinobacillus were obtained from the purulent content of the lung nodules. Similar findings were made on the other inoculated animal upon necropsy five months following infection.

Fig. 3—Bovine lung three months following subcutaneous inoculation of Actinobacillus lignieresi, strain FM. Numerous minute, shotlike, yellowish white nodules are present.

Three other cattle were inoculated intramuscularly into the tongue, hard palate, and cheeks with 1.00 ml. in each respective site of the described inoculum. Local lesions were not observed and these animals remained in good health until the experiment was terminated 6 months following inoculation.

Numerous attempts failed to infect rabbits, guinea pigs, and hamsters using subcutaneous and intraperitoneal routes of inoculation, and the Strauss testicular reaction was negative. When 4 hamsters, approximately 100 Gm. in weight, were inoculated with granular type of strain FM, 2 died at four days following inoculation; however, the organism was not cultured from these animals. A dozen hamsters were injected into the tongue and cheeks with 0.1 ml. of turbid suspension of strain FM. Abscesses formed at the site of injection in 24 hours but completely receded within two to four days. Mixed bacterial cultures were obtained from these abscesses, but Actinobacillus was not isolated. The chicken embryo proved to be highly susceptible to infection upon inoculation with most of the strains studied. Results of such infections with three colonial types of strain FM are presented (table 3). While the fluorescent type was less active in persisting and producing death in the chicken embryo, the granular and dwarf types were capable of doing so. With three exceptions, the variants were recovered from infected embryos of the same colonial type as inoculated.

Histopathology.—Microscopic appearance of cervical granulomatous lesions of naturally and artificially infected cattle revealed numerous necrotic foci, containing one or more smaller rosettes, surrounded by inflammatory cells and granulation tissues (fig. 4). Giant cells were not found in any of the sections studied. More detailed descriptions of such microscopic lesions have been reported.[4, 5, 14, 16] Liver lesions of experimentally infected chicken embryos were characterized by focal necrobiosis and lymphocytic infiltration of the sinusoides and central veins (fig. 5).

Antibiotics and Chemotherapeutics Sensitivity.—There was considerable similarity among the strains in regard to their sensitivity to different therapeutic agents (table 4). With the exception of strain 10811, which was sensitive, all others were resistant to bacitracin and magnamycin.[9] With slight variations, most strains were more resistant to viomycin, erythromycin, gantrisin, sulfadiazine, and "triple-sulfa." There was no discrepancy in the sensitivity demonstrated among the strains to all three concentrations of chloromycetin,[9] aureomycin,[9] and terramycin.[9] Slight variations in degree of sensitivity among strains to the remaining antibiotics were demonstrated.

Discussion

Examination of 14 cultures of *A. lignieresi* demonstrated that all contained at least two of the described colonial variants,

TABLE 3—Survival of 11-Day-Old Chicken Embryos Inoculated with Varying Numbers of Variants of Actinobacillus Lignieresi (Strain FM)

Variant type	Number of organisms	Death at days					Total deaths	Cultures of survivors	
		1	2	3	4	5			
Fluorescent	3.4×10^7	1	2	0	0	0	3/5	1+	1−
	3.4×10^6	1	0	1	0	0	2/5	1+	2−
	3.4×10^5	2	0	0	0	0	2/5	1+	2−
	3.4×10^4	1	1	0	0	0	2/5	3−	
	3.4×10^3	0	0	0	1	0	1/5	4−	
	3.4×10^2	0	0	0	0	0	0/5	5−	
Granular	3.7×10^7	2	3	–	–	–	5/5	....	
	3.7×10^6	2	2	1	–	–	5/5	....	
	3.7×10^5	2	1	1	1	–	5/5	....	
	3.7×10^4	1	2	2	–	–	5/5	....	
	3.7×10^3	1	1	1	1	0	4/5	1+	
	3.7×10^2	0	1	2	1	0	4/5	1+	
Dwarf	4.2×10^7	3	2	–	–	–	5/5	....	
	4.2×10^6	2	1	1*	1*	–	5/5	....	
	4.2×10^5	1	1	2	1	–	5/5	....	
	4.2×10^4	2	2	1	–	–	5/5	....	
	4.2×10^3	0	2	2	0	1*	5/5	....	
	4.2×10^2	0	1	1	2	1	5/5	....	

Inoculations were via chorioallantoic cavity with 0.2 ml. of bacterial suspension.
* Organisms recovered were in the granular colony form. All dead embryos yielded cultures in the same colonial form as inoculated.

TABLE 4—Sensitivity of Actinobacillus Lignieresi to Antibiotics and Chemotherapeutics

	Strain numbers												
	G	FM	90	N	10811	32774	1185	33443	208	209	210	211	Dwarf
Chloromycetin®	S	S	S	S	S	S	S	S	S	S	S	S	S
Aureomycin®	S	S	S	S	S	S	S	S	S	S	S	S	S
Terramycin®	S	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S	S	S	R
Polymyxin B	S	S	S	S	SL	S	S	S	S	S	S	SL	R
Penicillin	SL	SL	SL	SL	SL	SL	SL	S	S	S	S	SL	S
Streptomycin	SL	SL	SL	SL	SL	SL	SL	S	S	S	S	S	S
Dihydro Streptomycin	SL	SL	SL	SL	SL	S	S	S	S	SL	S	SL	S
Neomycin	SL	SL	SL	SL	SL	S	SL	S	S	SL	S	SL	S
Erythromycin	SL	SL	R	R	S	SL	SL	SL	SL	SL	S	SL	R
Bacitracin	R	R	R	R	S	R	R	R	R	R	R	R	R
Viomycin	SL	SL	R	R	R	SL	R	SL	S	SL	SL	SL	SL
Gantrisin	R	R	R	R	R	S	R	R	SL	SL	R	R	R
Sulfadiazine	R	SL	R	R	R	S	S	R	S	S	R	R	R
Triple sulfa	R	SL	SL	R	R	R	S	SL	S	SL	R	R	R
Magnamycin®	R	R	R	R	S	R	R	R	R	R	R	R	S

Standard Difco bacterial sensitivity discs of three concentrations were employed.

S Sensitive, clear zones around all three concentrations; SL slightly sensitive, completely resistant to lowest concentrations; slightly, or not sensitive, to middle; sensitive to highest concentrations; R resistant, no zones around any concentration.

but that their ratio varied with the strain. An attempt was made to determine the age and source of the strain as factors influencing variation. While the dwarf forms were present in freshly isolated cultures only, and while the fluorescent type was predominant in stock cultures, proportions of granular and fluorescent type in a given culture was not determined solely by the age of the strain. Although those cultures which had been recently isolated consistently showed a greater number of granular variants, the granular type was also found in some of the older cultures. On the basis of colonial variability, differentiation between strains of ovine and bovine origin was not possible. The use of blood serum agglutination test for diagnosis of actinobacillosis has been described by Magnusson.[11] A study of such a possibility should be made by evaluating the antigenic properties of the variants. Such a study is now in progress in our laboratories.

The important differences between the

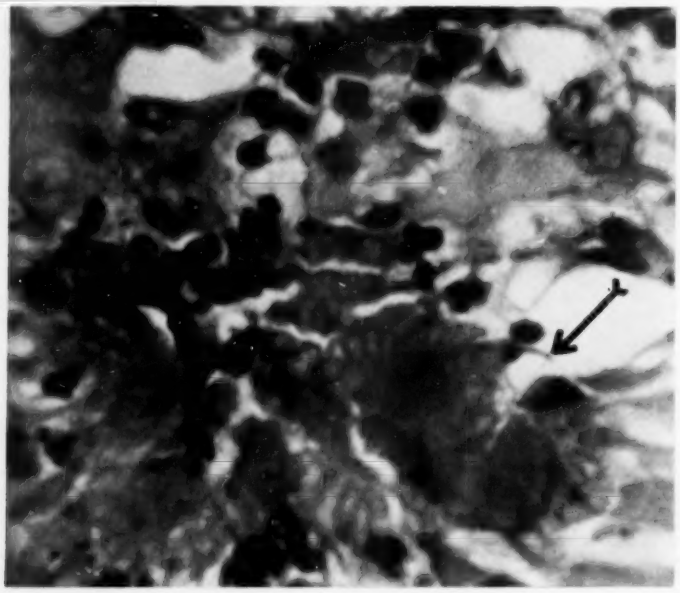

Fig. 4—Club-bearing rosettes (arrow) surrounded by lymphocytes and granulating tissue from one of the cattle naturally infected with Actinobacillus lignieresi.

TABLE 5—Comparison of Activities of Actinobacillus Lignieresi Strains

Strains	Acid produced from			Indol produced	Nitrate reduced	Urea attacked	H_2S produced
	Fructose	Sucrose	Mannitol				
Bergey's description	+	+	+	+ Trace	—	+	*
A.T.C.C. strain 10811	—	—	—	+ Trace	—	—	+
Other strains	+[x]	+	+	—	+	+	Trace or Negative

* No information; + activity; — no activity; [x] dwarf strain produced acid from fructose.

A.T.C.C. strain 10811, all other strains studied, and the description of actinobacillus presented in Bergey's Manual [2] are presented (table 5). It can be seen that the characteristics of the A.T.C.C. strain differ from the other two descriptions in regard to their sugar reactions. On the other hand, its characteristics agree with Bergey's Manual in regard to indol production and nitrate reduction, while all other strains studied differed in these characteristics. The ability to attack urea and produce H_2S is not described in Bergey's Manual and the A.T.C.C. strain differs from all other strains studied in these activities. Age of the strain does not seem to be a factor influencing the difference between the A.T.C.C. strain and others, since strains of equal age did not resemble it. Whether the differences observed were inherent in the culture at the time of isolation or were a result of the selection of mutants during the long period of maintenance on artificial mediums can only be a subject of speculation. In view of these findings it appears that a re-evaluation of the description of *A. lignieresi* as found in Bergey's Manual should be considered.

Opinions have been expressed by a number of investigators that the natural portal of entry for Actinobacillus may be the mucous membrane of the oral cavity. Such an hypothesis was not supported by the limited data obtained from artificial infections in these experiments. However, animals infected via oral mucous membrane had been under clinical observations only and

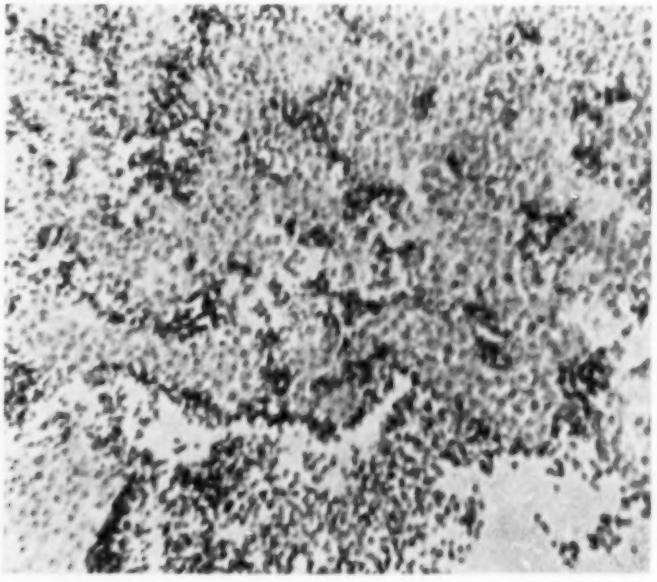

Fig. 5—A section from the liver of an 11-day chicken embryo experimentally infected with Actinobacillus lignieresi, strain G. Parenchymal destruction and inflammatory cell infiltration are prominent.

the possibility of internal subclinical infections exists.

The long incubation period as observed in the successfully infected cattle is of interest; however, this period was predicated on the appearance of subcutaneous granulomas. We have no evidence whether or not lung lesions existed before this time. The animals were apparently in good health until approximately the time of the clinical appearance of the granulomas. At that time the variational phenomenon had not been studied.

It appears that the fluorescent type, which tends to predominate in stock cultures, is less pathogenic as measured by chicken embryo lethality. Thus the possibility arises that cattle might have been less susceptible to this variant which could account for the lack of pathogenicity. Originally, Lignieres described the organism as pathogenic for laboratory animals,[9] while Topley and Wilson [21] failed to demonstrate this characteristic. This might have been due to the use of freshly isolated cultures by Lignières, in which the pathogenic variants were present. At the present time, the chicken embryo apparently is the only susceptible laboratory animal which may be employed in experimental studies of actinobacillosis. Studies on the pathogenicity of the colonial variants for laboratory animals are now in progress.

The results of *in vitro* sensitivity of Actinobacillus for antibiotics and chemotherapeutic agents offer a general qualitative guide to therapy. The lowest concentrations of aureomycin, terramycin, and chloromycetin consistently inhibited the growth of all strains. The effectiveness of these drugs must be studied *in vivo* before their therapeutic efficiency can be determined.

SUMMARY

1) A study was made of 14 strains of *Actinobacillus lignieresi* obtained from various stock collections and also organisms freshly isolated from cattle in Florida.

2) Study of colonial characteristics revealed the presence of the dwarf, granular, and fluorescent variants.

3) All strains studied behaved alike in their biochemical characteristics, with the exception of the A.T.C.C. strain 10811, in regard to activity on fructose, xylose, sucrose, mannitol, indol, urease, and H_2S production and nitrate reduction.

4) None of the strains studied completely matched the description presented in Bergey's Manual.

5) The 11-day-old chicken embryo was found to be susceptible to infection; guinea pigs, hamsters, and rabbits proved to be resistant; clinical bovine infections were induced by use of subcutaneous inoculations.

6) The strains studied proved to be uniformly susceptible to aureomycin,® terramycin,® and chloromycetin® as demonstrated *in vitro*.

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Serological Cross-Agglutination Reactions Between *Leptospira Pomona* and *Leptospira Icterohaemorrhagiae*, AB

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EARLY detection of the leptospiroses and rapid identification of the responsible serotype is essential. *Leptospira pomona* has been incriminated as the cause of the vast majority of outbreaks of bovine and porcine leptospirosis in North America. *Leptospira icterohaemorrhagiae*, however, has been responsible for some infections in calves[3,6] and in pigs.[4,11] Leptospirosis attributable to the latter microorganism apparently either is not a common occurrence in cattle or swine, or it remains unrecognized in the United States.

The serological cross reactivity of *Leptospira canicola* and *L. icterohaemorrhagiae* is well known. Evidence indicates[5,15] that some antigenic relationship may exist between some strains of *L. pomona* and *L. icterohaemorrhagiae* (AB) as well. Serological reactions between these two serotypes may lead to confusion in the diagnosis of the respective leptospiroses. It was observed during routine serological examinations that a number of cattle naturally infected with *L. pomona* in Wisconsin developed concurrent titers for *L. icterohaemorrhagiae* (AB). This finding served as a stimulus for the investigations reported here.

MATERIALS AND METHODS

The materials examined were as follows: (1) serums from cattle which were naturally infected with *L. pomona*;[7,8] (2) serums of cattle, swine, and a goat which were infected experimentally with *L. pomona* (strain Wickard);[9,10] and (3) serums of guinea pigs and hamsters which were inoculated intraperitoneally with tissues from animals infected with *L. pomona*.

The microscopic agglutination-lysis tube test was employed, using living leptospiral antigens. In the routine serological studies,[8] sterile 0.85 per cent sodium chloride solution was employed in the tests to dilute the serums of naturally infected cattle. It was later found, however, that a sterile buffer solution used as the serum diluent produced fewer "breed nests," *i.e.*, aggregates of leptospiral cells which could be mistaken for agglutinated cells. The buffer consisted of Na_2HPO_4, 2 Gm., KH_2PO_4, 0.4 Gm., and 4 Gm. of NaCl in 1 liter of distilled water, and had a final pH of 7.0. The agglutination-lysis tests using buffered diluent were conducted on the serums from the animals experimentally infected with *L. pomona* and from the infected guinea pigs and hamsters. Four antigens were used in the investigation: (1) *L. pomona* (strain Johnson), and (2) *L. icterohaemorrhagiae* (AB) were obtained in 1952 from Dr. K. R. Reinhard, U. S. Public Health Service, Rocky Mountain Laboratory, Hamilton, Mont.; (3) *L. pomona* ("media" strain Wickard); and (4) *L. pomona* ("animal" strain Wickard) were isolated by the authors[7] by means of guinea pig inoculation from the urine of an infected dairy cow in Wisconsin. The media strain of *L. pomona* (Wickard) was cultivated by serial transfers in Chang's fluid medium,[2] containing 0.01 per cent hemoglobin (Difco), for over two years. The mammalian serum, which was added to give a final concentration of 10 per cent of the medium, did not contain agglutinins for any of the common leptospiral serotypes employed in routine diagnostic work at the State Laboratory of Hygiene.[7] The animal strain of *L. pomona* (Wickard) had been maintained continuously in young guinea pigs for the same period. It had received a passage in a calf just prior to the initiation of the experiment and had been subjected to only two passages in Chang's medium[2] in preparation for its use as antigen in the agglutination-lysis tests.

Leptospiral cells for serum absorption were obtained by propagating the microorganisms in Roux bottles containing approximately 60 ml. of Chang's fluid medium.[2] Optimal growth occurred within seven days at 28 to 30 C. When such was observed, the cultures were placed in the refrigerator at 4 C. for 72 hours in order to kill the leptospiras. The cells were concentrated by centrifugation. The cell suspension was added to the bovine serum so that a 1:5 dilution of the serum

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resulted. The serum-cell suspension was incubated at 37 C. in a water bath for one hour and then placed in the refrigerator at 4 C. for 16 to 20 hours in order to complete the absorption. The cells were then removed by centrifugation and the clear, diluted, supernatant serum was used in the agglutination-lysis tests. The final serum dilutions employed were 1:10, 1:50, 1:100, 1:500, and 1:1000. These dilutions, as expressed, did not include the dilution factor created by the addition of antigen to each tube.

RESULTS

Leptospira icterohaemorrhagiae (AB) agglutinin-lysins were demonstrated in the blood of 183 of the 219 cattle with serological reactions for *L. pomona*. Approximately 47 per cent fo the total number of cattle in 11 herds infected with *L. pomona* had a demonstrable heterologous titer of *L. icterohaemorrhagiae*, while 56 per cent were found to have the homologous or *L. pomona* titers. Among the 183 cattle which were found to have *L. icterohaemorrhagiae* serum titers, 29 per cent were at 1:10,000, 23 per cent at 1:1000, 25 per cent at 1:100, and 23 per cent at 1:10. The percentages of the animals having *L. pomona* titers and their distribution at the various levels were 78 per cent at 1:10,000, 11 per cent at 1:1000, 5 per cent at 1:100, and 6 per cent at 1:10 (table 1).

Less than 0.1 per cent of the cattle in herds infected with *L. pomona* were found to have *L. icterohaemorrhagiae* (AB) serological reactions in the absence of *L. pomona* titers. The heterologous agglutinins were present at no higher than the 1:10 serum dilution. Among 155 cattle which had an *L. pomona* reaction of 1:10,000, only 8 per cent did not have a demonstrable heterologous titer. The distribution of *L. icterohaemorrhagiae* titers among cattle in 12 herds infected with *L. pomona* is shown (table 2).

TABLE 2—Distribution of Agglutinin-Lysin Titers for Leptospira Icterohaemorrhagiae (AB) Among Cattle in 12 Herds Which Were Naturally Infected with Leptospira Pomona

L. pomona serum titer	No. samples	*L. icterohaemorrhagiae* (AB) serum titer				
		0*	1:10	1:100	1:1000	1:10,000
0*	156	154	2			
1:10	21	18	3			
1:100	14	6	3	5		
1:1000	26	6	9	8	3	
1:10,000	155	12	26	31	41	45

* No evidence of agglutination or lysis at 1:10 serum dilution.

Leptospira icterohaemorrhagiae serum titers developed in guinea pigs and in hamsters which were inoculated with blood, urine, or tissues obtained from animals infected with *L. pomona*. Three of ten serums which were negative for *L. pomona* agglutinins gave reactions with *L. icterohaemorrhagiae* antigen. Evidences of heterologous titer response, which was observed in the laboratory animals, were not as significant as the results obtained in naturally infected cattle. The distribution of homologous and heterologous serum titers in guinea pigs and hamsters is shown (table 3).

Serums from a group of cattle experimentally infected with *L. pomona* (Wickard) were examined by the microscopic agglutination test employing formolized leptospiral antigens.* The results also in-

* The tests were made by Mrs. Mildred Galton at the Communicable Disease Center, Public Health Service Laboratory, Chamblee, Ga.

TABLE 1—Distribution of Leptospira Pomona and Leptospira Icterohaemorrhagiae (AB) Serum Agglutinin-Lysins Among Cattle in Herds Infected with L. Pomona

Herd	Approximate months following onset	*L. pomona* titer distribution					*L. icterohaemorrhagiae* (AB) titer distribution				
		0*	1:10	1:100	1:1000	1:10,000	0*	1:10	1:100	1:1000	1:10,000
Grade H.-F.	3	15	0	1	0	12	17	0	2	2	7
Grade H.-F.	1	0	0	1	3	17	1	1	4	11	4
	5	16	3	1	4	22	20	20	6	0	0
Purebred H.-F.**	2	10	2	0	1	2	12	2	0	0	1
Purebred Guernsey	2	11	2	1	1	8	15	0	3	2	3
Grade H.-F.	1	4	2	0	0	13	5	1	3	2	8
Grade H.-F.	2	7	0	0	1	11	7	2	4	1	5
Grade H.-F.	1	7	2	1	2	16	13	0	5	8	2
Grade H.-F.** †	1	4	0	0	2	24	4	4	9	3	10
Purebred Angus	6	83	1	5	6	11	94	0	3	5	4
Grade H.-F.**	3	5	2	0	2	21	9	9	6	5	1
Grade H.-F.**	1	9	0	1	1	14	10	1	1	4	9
	Totals:	171	14	11	23	171	207	40	46	43	54

H.-F.—Holstein-Friesian; * no evidence of agglutination or lysis at 1:10 serum dilution; ** *L. pomona* isolated from cow's urine in this herd; † herd which yielded strain Wickard.

dicated the relationship of the Wickard strain of *L. pomona* to a typical representative of this serotype and to two strains of *L. icterohaemorrhagiae* (AB) (table 4).

During the course of experimental *L. pomona* infection in cattle, pigs, and a goat, serum agglutinin-lysins also appeared for *L. icterohaemorrhagiae*.[10] The simultaneous occurrence, in most instances, of reactions for both serotypes was observed. With one serum sample collected from cow 2 at 13 days following exposure, the heterologous titer exceeded that of the homologous titer (table 5). By the fifteenth day following exposure, the heterologous titers were generally lower than those observed for homologous serotype. The *L. icterohaemorrhagiae* titer remained demonstrable throughout the 48 days following exposure. The majority of the maximal heterologous responses occurred between the twentieth and thirtieth days of infection. Since the experiments were terminated at 48 days, the duration of persistence of the heterologous titer is not known. The pertinent data are shown (table 5).

Leptospira icterohaemorrhagiae agglutinin-lysins were removed from reactive bovine serums by absorption with this serotype; a similar effect could be produced by one or more absorptions with *L. pomona; L. icterohaemorrhagiae* absorption resulted in a decrease in titer for *L. pomona* in the majority of cases. In most of the examinations, the media strain of *L. pomona* (Wickard) proved to be less reactive with the serums than was the animal strain of *L. pomona* (Wickard) as shown by lower titer end points for the former. Two absorptions with the media strain followed by an absorption with *L. pomona* (Johnson) were incapable of completely removing agglutinin-lysins for the animal strain of *L. pomona* (Wickard). These examinations were repeated several times and agglutinins for the animal strain persisted in the serums of 2 cows (53 and 045). The results of the agglutinin absorption tests are given (table 6).

TABLE 3—Distribution of Agglutinin-Lysin Titers for Leptospira Icterohaemorrhagiae (AB) Among Guinea Pigs and Hamsters Exposed via the Intraperitoneal Route to Leptospira Pomona

L. pomona serum titer	No. samples	*L. icterohaemorrhagiae* (AB) serum titers				
		0*	1:10	1:100	1:1000	1:10,000
0*	10	7	2**	1		
1:10	2		1**	1**		
1:100	7	3	2	2		
1:1000	4		1**	3		
1:10,000	36	6**	13	15	2	

* No evidence of agglutination or lysis at 1:10 dilution; ** 1 hamster serum sample, all other are guinea pig samples.

Discussion

The following serotypes of Leptospira are present in the United States as proved by their isolation from infected materials from man and from the lower animals: *L. icterohaemorrhagiae, L. canicola, L. pomona, L. ballum,* and *L. autumnalis.* Reinhard [13] indicates that additional serological evidence discloses the possible presence of *L. grippotyphosa* in man, cattle, and horses, *L. bataviae* in man, and *L. sejroe* in cattle. It is doubtful that this country can remain long with only five of these leptospiras considering the rapidity of global transportation today. Most of the species have been found to have a natural or reservoir host, e.g., *L. icterohaemorrhagiae* in rats, *L. ballum* in mice, *L. grippotyphosa* in voles, and *L. canicola* in dogs. Often

TABLE 4—Cross-Agglutinations* Among Serums of Heifers Experimentally Infected with Leptospira Pomona (Wickard)

Heifer	Agglutinin titer using antigen			
	L. pomona Wickard (media)	*L. pomona* 891 (CDC strain)	*L. icterohaemorrhagiae* AB (Wis. antigen)**	*L. icterohaemorrhagiae* AB (Wijnberg strain)
1	1:1024	1:512	1:64	1:64
2	1:512	1:128	1:32	1:16
3	1:4096	1:2048	1:64	1:64
4	1:4096	1:2048	1:64	1:64
6	1:1024	1:1024	1:64	1:32
7	1:1024	1:1024	1:16	1:16

* Microscopic agglutination test using formolized leptospiral antigens. Conducted at the Communicable Disease Center, Chamblee, Ga., by Mrs. Mildred Galton and Mrs. D. K. Powers.

** Strain used for routine serological diagnostic work; originally obtained from Dr. K. R. Reinhard, U. S. Public Health Service.

TABLE 5—Serological Results with Serums of Animals Experimentally Exposed to Leptospira Pomona (Wickard)

Animal	Antigen	Serum dilution* at days** (2–6)	(7–9)	(11–12)	(13)	(15–16)	(17–18)	(20–25)	(26–30)	(31–37)	(40–48)
Cow 1	*L. pomona*	0†	1:10	$1:10^3$, $1:10^4$							
	L. icterohaemorrhagiae	0	1:10	$1:10^3$, $1:10^4$							
Cow 2	*L. pomona*	0	1:10	1:10	$1:10^3$			$1:10^4$, $1:10^4$			
	L. icterohaemorrhagiae	0	0	1:10	$1:10^3$			$1:10^3$, $1:10^3$			
Cow 3	*L. pomona*	0	1:10	$1:10^3$	$1:10^4$			$1:10^4$, $1:10^6$	$1:10^6$		
	L. icterohaemorrhagiae	0	0	$1:10^3$	$1:10^3$			$1:10^3$, $1:10^3$	$1:10^3$		
Cow 4	*L. pomona*	0	1:10	$1:10^3$	$1:10^3$			$1:10^4$, $1:10^4$	$1:10^6$		$1:10^6$
	L. icterohaemorrhagiae	0	1:10	$1:10^3$	$1:10^3$			$1:10^3$, $1:10^3$	$1:10^3$		$1:10^5$
Cow 5	*L. pomona*	0	1:10								
	L. icterohaemorrhagiae	0	0								
Cow 6	*L. pomona*	0	1:10	1:10	$1:10^2$	$1:10^4$	$1:10^4$	$1:10^4$, $1:10^3$	$1:10^4$	$1:10^5$	
	L. icterohaemorrhagiae	0	0	0	0	0	0	0, 1:10	$1:10^3$	$1:10^5$	
Cow 7	*L. pomona*	0	1:10	$1:10^2$	$1:10^2$	$1:10^3$	$1:10^4$	$1:10^5$, $1:10^4$	$1:10^5$	$1:10^5$	$1:10^3$, $1:10^4$
	L. icterohaemorrhagiae	0	0	0	0	$1:10^2$	$1:10^2$	$1:10^2$, $1:10^2$	$1:10^3$	$1:10^3$	$1:10^3$, $1:10^3$
Cow 8	*L. pomona*		0	1:10			$1:10^2$		$1:10^2$, $1:10^2$	$1:10^5$	
	L. icterohaemorrhagiae		0	0			$1:10^2$		$1:10^2$, $1:10^2$	$1:10^2$	
Cow 9	*L. pomona*		0	0			1:10		$1:10^2$, $1:10^2$	$1:10^3$	
	L. icterohaemorrhagiae		0	0			0		1:10, 1:10	1:10	
Cow 10	*L. pomona*		0	0			1:10	$1:10^3$			
	L. icterohaemorrhagiae		0	0				$1:10^4$			
Cow 11	*L. pomona*		0	0			1:10	$1:10^3$	$1:10^3$		
	L. icterohaemorrhagiae		0	0			1:10	$1:10^2$	$1:10^4$		
Calf 1	*L. pomona*		0	$1:10^2$							
	L. icterohaemorrhagiae		0	1:10							
Calf 2	*L. pomona*		0	$1:10^2$		$1:10^2$		$1:10^2$		$1:10^3$, $1:10^3$	$1:10^3$
	L. icterohaemorrhagiae		0	1:10		1:10		$1:10^2$		$1:10^2$, 1:10	1:10
Calf 3	*L. pomona*		1:10			$1:10^2$		$1:10^2$			
	L. icterohaemorrhagiae		0			1:10		$1:10^2$			
Calf 4	*L. pomona*		$1:10^2$			$1:10^2$		$1:10^4$	$1:10^6$	$1:10^5$	$1:10^5$, $1:10^5$
	L. icterohaemorrhagiae		1:10			$1:10^2$		1:10	$1:10^3$	$1:10^3$	$1:10^3$, $1:10^3$
Calf 5	*L. pomona*		$1:10^2$			$1:10^4$		$1:10^4$	$1:10^4$	$1:10^5$	$1:10^5$, $1:10^4$
	L. icterohaemorrhagiae		1:10			$1:10^2$		$1:10^2$	$1:10^3$	$1:10^3$	$1:10^3$, $1:10^2$
Calf 6	*L. pomona*		$1:10^2$			$1:10^2$		$1:10^5$	$1:10^6$	$1:10^5$	$1:10^5$, $1:10^5$
	L. icterohaemorrhagiae		1:10			$1:10^2$		$1:10^4$	$1:10^2$	$1:10^2$	$1:10^3$, $1:10^3$
Pig 1	*L. pomona*		0			$1:10^4$		$1:10^5$			
	L. icterohaemorrhagiae		0					$1:10^5$			
Pig 2	*L. pomona*		0					$1:10^5$		$1:10^5$	
	L. icterohaemorrhagiae		0					$1:10^2$		$1:10^3$	
Goat 1	*L. pomona*		0			$1:10^3$					
	L. icterohaemorrhagiae		0			1:10					

* Agglutination-lysis test using *L. pomona* (Johnson) and *L. icterohaemorrhagiae* (AB) as living cell antigens.

** Approximate days following subcutaneous exposure (cows 1–7 and calves 1–6) or contact with infected calves as exposure (cows 8–11, pigs, and goat) to *L. pomona* (Wickard).

† No evidence of agglutination or lysis at the 1:10 serum dilution.

these infections are rather benign or subacute or undetected clinically in the natural hosts. Man and his domestic animals, which might be considered the aberrant or accidental hosts for these microorganisms, may suffer a severe and sometimes fatal outcome of infection. The antigenic relationships of these serotypes to one another is of the utmost importance and bears directly upon their ultimate detection and recognition as distinct etiological entities in the infections of man and animals.

Wolff and Broom [15] investigated the serological relationships of 36 leptospiral strains or serotypes collected from all parts of the world and found an antigenic relationship between *L. pomona* and *L. icterohaemorrhagiae* (AB). The cattle infected with *L. pomona* and which concurrently developed serological titers for *L. icterohaemorrhagiae* (AB) presented a problem in serodiagnosis. The use of a single leptospiral agglutination-lysin test antigen in the examination of serum is not a judicious laboratory approach. Because of high interagglutinability among serotypes, e.g., 1:10,000 titers for *L. icterohaemorrhagiae* in *L. pomona* infected animals, a single test utilizing the antigen of only one serotype would indicate infection with the related but heterologous strain.

On the other hand, if an unrelated antigen was employed, the presence of leptospirosis would be negated by the absence of agglutination. The complement-fixation test has been proposed to screen serums for group antigen.[12] The erythrocyte sensitizing substance test of Chang and McComb [1] also provides a group of generic antigen but, unfortunately, the antigen does not remain fully active for longer than a few months when stored at 0 to 4 C. At the present time, most laboratories rely on some modification of the more specific agglutination or agglutination-lysis tests. Under the circumstances, a possible solution might be first to examine bovine and porcine serum samples by one of the agglutination tests for *L. pomona*. If inconclusive or negative serological results occur, the serums should be examined for the presence of agglutinins for *L. icterohaemorrhagiae*, *L. canicola*, *L. sejroe*, and *L. grippotyphosa*. The latter tests should be conducted only when there is clinical evidence highly suggestive of leptospirosis.

TABLE 6—Agglutinin-Absorption Tests Conducted with Serums from Cattle Experimentally Infected with Leptospira Pomona (Wickard)

Serum	Absorbing serotype	Titer against antigens: *L. pomona* Wickard (media)*	*L. pomona* Wickard (animal)*	*L. pomona* Johnson	*L. icterohaemorrhagiae* (AB)
Cow 19 (14 days following exposure)	None	1:50 P**	1:100 ±	1:500 ±	1:100 ±
	L. pomona, Wickard (media)*	Neg.	1:10 ±	1:100 ±	Neg.
	L. icterohaemorrhagiae (AB)	1:10 P	1:50 P	1:100 ±	Neg.
Cow 54 (21 days following exposure)	None	1:1000 ±	1:1000 P	1:1000 ±	1:500 P
	L. pomona, Wickard (media)	Neg.	1:10 ±	1:100 ±	1:10 P
	L. icterohaemorrhagiae (AB)	1:100 P	1:100 P	1:500 ±	Neg.
Cow 54 (48 days following exposure)	None	1:500 ±	1:1000 ±	1:500 ±	1:100 P
	L. pomona, Wickard (media)	Neg.	1:50 ±	1:50 ±	Neg.
	L. icterohaemorrhagiae (AB)	1:100 P	1:500 ±	1:500 ±	Neg.
Cow 53 (13 days following exposure)	None	1:500 ±	1:1000 ±	1:500 ±	1:50 ±
	L. pomona 1st: Wickard (media) 2nd:	1:10 ±	1:100 ±	1:100 ±	1:10 ±
	L. pomona (Johnson)	1:10 +P	1:100 ±	1:100 ±	1:10 ±
	L. icterohaemorrhagiae (AB)	Neg.	1:10 ±(?)	Neg.	Neg.
Cow 045 (20 days following exposure)	None	1:500 ±	1:1000 ±	1:500 ±	Neg.
	L. pomona 1st: Wickard (media) 2nd:	1:1000 P	1:1000 ±	1:1000 P	1:50 ±
	L. pomona (Johnson)	1:10 +P	1:100 ±	1:100 ±	Neg.
	L. icterohaemorrhagiae (AB)	Neg.	1:10 ±(?)	Neg.	Neg.
		1:500 P	1:500 ±	1:500 P	Neg.

* *L. pomona*, Wickard (media) = cultivated in Chang's fluid medium for over two years following isolation from cow urine. (Animal) = same strain, but passage maintained in guinea pigs, except for two transfers in mediums to prepare antigen for tests.

** Serum dilution 1:10, 1:50, 1:100, 1:500, and 1:1000 and living antigens employed.

±25 per cent agglutination or lysis; P 50 per cent agglutination of lysis; +P 75 per cent agglutination or lysis.

Serological reexaminations at two- to four-week intervals will indicate, in the case of concurrent titers, which of two serotypes is the infecting microorganism. Eventually, the homologous titer will exceed that of the heterologous serological reaction; however, this may require seven to eight weeks of time following the onset of infection.[5]

The specificity of the observed serum agglutinin-lysins for *L. icterohaemorrhagiae*, which developed in the animals infected with *L. pomona*, is questionable. The leptospiral coagglutinins of Wiesman[14] were both specific and nonspecific in nature. The latter do not correspond to any antigenic fraction of the heterologous serotypes; they appear early in the course of the disease and gradually disappear as the infection progresses; upon storage of serums in the fluid state, the nonspecific agglutinins disappear readily while little decrease in reactivity is observed with the specific coagglutinins. The fact remains, however, that the cross reactions which were observed and reported can cause confusion in the serodiagnosis of the leptospiroses. Further investigations as to the specific or nonspecific nature of the *L. icterohaemorrhagiae* (AB) agglutinins are contemplated.

SUMMARY

Leptospira icterohaemorrhagiae (AB) agglutinins and lysins were found in 183 serums from 219 cattle naturally infected with *L. pomona* and which developed titers for the infecting microorganism. In general, the homologous or *L. pomona* titer exceeded the heterologous or *L. icterohaemorrhagiae* titer. Two of 156 cattle were found to have a heterologous titers but a homologous reaction was absent. Heterologous serotype titers were not detectable in the serums of all cattle with demonstrable *L. pomona* titers.

The cross-agglutination reaction was also observed in the serums of guinea pigs and hamsters, which were inoculated with *L. pomona* infected material.

Cattle, swine, and a goat concurrently developed serum titers for the two serotypes during the experimental *L. pomona* infection. Titers persisted for both leptospiral species for 48 days, at which time the experiments were concluded.

When the serums of the cattle, which were experimentally infected with *L. pomona*, were absorbed with *L. icterohaemorrhagiae* (AB), all reactivity for *L. icterohaemorrhagiae* was removed and that for *L. pomona* was decreased. Evidence indicated that *L. pomona* (Wickard) which had been propagated in mediums for over two years, may have undergone a minute antigenic change.

The implications of these findings with respect to the serodiagnosis of leptospiroses are discussed.

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